

**Development and Characterisation
of a Portland cement-based
Dental Root Filling Material**

by

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ABSTRACT

Mineral trioxide aggregate (MTA) is a Portland cement (PC)-based endodontic material used for sealing root canals. This study investigated the effect of calcium sulphate additions for improving the undesirably-long setting time of MTA-like dental materials, whilst maintaining the mechanical, biological and sealing properties.

10wt%PoP accelerated initial setting times of grey and white model cements and MTA from >6h to <40min, and did not significantly change compressive strengths and relative porosities with long-term storage in media. Cement pastes containing PoP may 'false' set or stiffen through gypsum precipitation, seen in scanning electron photomicrographs of MTA-like cements with 30wt%PoP.

Similar *in vitro* responses of adult and neonatal BMSC, periosteal and osteoblastic cultures were noted with PoP-modified and unmodified cements. Inhibition of cell growth was seen with 3day-cultures containing modified model cements and MTA, the possible result of calcium hydroxide release from cements.

Sealing properties were characterised using dye leakage studies and concluded that the sealing abilities of model cements and MTA were not compromised by PoP addition.

In summary, 10wt%PoP has shown potential as a modification to MTA by reducing the setting time whilst maintaining mechanical stability, solubility, *in vitro* responses to and the sealing properties of MTA, therefore, warrants further investigation.

Dedicated to
Eileen O'Beirne
(Nan)

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LIST OF ACRONYMS

Bi ₂ O ₃	Bismuth oxide
Bi ₂ O ₃ (Sigma)	Bismuth oxide manufactured by Sigma Aldrich
Bi ₂ O ₃ (Acros)	Bismuth oxide manufactured by Acros Organics
BMSC	Bone marrow stromal cells
CaSO ₄	Anhydrous calcium sulphate
CaSO ₄ (Acros)	Anhydrous calcium sulphate manufactured by Acros Organics
CaSO ₄ (Sigma)	Anhydrous calcium sulphate manufactured by Sigma Aldrich
Ca(OH) ₂	Calcium hydroxide
CS	Compressive strength
CSH	Calcium silicate hydrate - rigid form
C-S-H	Calcium silicate hydrate - poorly crystalline
C ₃ A	Tricalcium aluminate (aluminate phase)
C ₂ S	Belite, dicalcium silicate
C ₃ S	Alite, tricalcium silicate
C ₄ AF	Calcium aluminoferrite
Gyp	Gypsum, calcium sulphate dihydrate
Gyp (Acros)	Gypsum manufactured by Acros Organics
Gyp (Sigma)	Gypsum manufactured by Sigma Aldrich
MC	Mastercrete (Portland cement)
MTA	Mineral trioxide aggregate
OPC	Ordinary Portland cement

PC	Portland cement
PLR	Powder to liquid ratio
PoP	Plaster of Paris, calcium sulphate hemihydrate
PoP + CaSO ₄	Plaster of Paris and anhydrous calcium sulphate both added
RP	Relative porosity
SC	Snowcrete (Portland cement)
SEM	Scanning electron microscopy
XRD	X-ray diffraction

CONTENTS

1	INTRODUCTION	1
1.1	Structure of the tooth	23
1.2	Root canal treatment: the need for root canal filling materials.....	25
1.3	Limitations of root filling materials: introduction of mineral trioxide aggregate.....	25
1.4	Portland cement	27
1.5	The hydration of Portland cement	29
1.5.1	Hydration of calcium silicate phases: alite and belite	29
1.5.2	Hydration of aluminate phases: tricalcium aluminate	30
1.5.3	Final phases formed during setting.....	32
1.5.4	Abnormal setting of Portland cement: ‘Flash’ setting.....	32
1.5.5	Abnormal setting of Portland cement: ‘False’ setting.....	32
1.6	Setting times of mineral trioxide aggregate (MTA) and Portland cement (PC).....	33
1.6.1	The effect of accelerants and modifications and the impact on CS.....	34
1.7	Biological properties of MTA and the effect of additives	36
1.7.1	<i>In vivo</i> studies	36
1.7.2	<i>In vitro</i> studies	38
1.7.3	<i>In vitro</i> and <i>in vivo</i> studies involving modified MTA and PC	43
1.8	Sealing properties of mineral trioxide aggregate, modified with additives	44
1.8.1	Dye/ink leakage	44
1.8.2	Bacterial leakage.....	46
1.8.3	Fluid filtration.....	47
1.8.4	Glucose and protein leakage.....	47
1.9	Antimicrobial properties of modified mineral trioxide aggregate	48
1.10	Other properties of mineral trioxide aggregate	49
1.10.1	Solubility and stability of mineral trioxide aggregate	50
1.11	Additives: Calcium sulphates.....	51
1.12	Aims of the project.....	51
2	MATERIALS AND METHODS	54
2.1	Cements and powders used.....	54
2.1.1	Powder and cement paste preparation	55
2.1.2	Sample generation	55
2.1.3	Sterilisation of powders and set cements.....	55
2.1.1	Samples for cell culture	56
2.2	Characterising cements	57
2.2.1	Setting time.....	57
2.2.2	Compressive strength	57
2.2.3	Weight loss experiment	58
2.2.4	Calculation of relative porosity	58

2.2.5	Scanning electron microscopy	59
2.2.6	X-ray diffraction analysis	60
2.2.7	Dissolution kinetics of the additives.....	60
2.3	Cell culture mediums and solutions	61
2.3.1	Medium.....	61
2.3.2	Cell extraction	62
2.4	Characterisation and generation of a control (model) cement	64
2.4.1	Selection of Portland cement for model system	64
2.4.2	Selection of bismuth oxide used as a component of the model system.....	65
2.4.3	Effect of time of mould storage on stability and structure of cement	65
2.5	Addition of calcium sulphates to the model system	66
2.5.1	Effect of additives on the microstructure of the model system	66
2.5.2	Selecting calcium sulphates.....	66
2.6	Addition of Plaster of Paris to mineral trioxide aggregate	66
2.7	Long term solubility and stability of the PoP-modified MTA-model	67
2.8	In vitro effect of the PoP-modified MTA-model	67
2.8.1	Cell morphology	67
2.8.2	Cell counts	68
2.8.3	Live/dead staining	69
2.8.4	Washing of cements to be placed into cell culture	69
2.9	Sealing ability of PoP-modified MTA and model cement	70
2.9.1	Establishing a method for dye leakage studies	70
2.9.2	Effect of dye permeation on PLR and cement composition.....	73
2.9.3	Tooth model for dye leakage studies.....	74
2.10	Statistical analysis of raw data	77

RESULTS..... 78

3	Characterisation and generation of a model system	78
3.1	SEM evaluation of cement powders	78
3.2	CS and RP of pure cements	80
3.3	Selection of the bismuth oxide component of the model system	82
3.4	Effect of mould storage time on stability and structure of cement	83
4	Incorporation of additives to the model cement and MTA	87
4.1	Microstructure of calcium sulphate powders	87
4.2	Modifying the model cement with calcium sulphates	88
4.3	Varying additive concentrations in the MC model cement	93
4.3.1	Effect of additives on initial setting times of model cement	93
4.3.2	CS and RP of model cements containing calcium sulphates.....	95
4.3.3	Examination of the set microstructure with SEM	98
4.4	Addition of calcium sulphates to Snowcrete-based model cements.....	103
4.4.1	Effect on initial setting times.....	103
4.4.2	Compressive strength and relative porosity	104
4.5	Plaster of Paris addition to mineral trioxide aggregate: effect on setting, CS and RP ..	106
4.5.1	Initial setting times of cements with PoP additions.....	106
4.5.2	CS and RP of MTA and the model system containing PoP	107

4.5.3	SEM examination of set structures	109
4.6	Analysis of the crystal structures of the modified model system and mineral trioxide aggregate using XRD	112
4.7	Kinetic solubility of calcium sulphate additives	115
5	Preliminary work: Sterilisation of powders/ set cements	116
6	Effect of ageing on the stability and solubility of modified cements	118
6.1	Long term stability of cements modified with Plaster of Paris.....	118
6.1.1	Effect of water storage on CS and RP of modified grey model cements	118
6.1.2	Effect of water storage on CS and RP of modified white model cements	120
6.1.3	Effect of storage in medium on CS and RP of modified grey model cements.....	122
6.1.4	Effect of storage in medium on CS and RP of modified white model cements	126
6.2	Long term solubility of the model system with PoP.....	128
6.2.1	Release of calcium ions from cement into water.....	128
6.2.2	SEM analysis of modified grey model cements	130
6.2.3	SEM analysis of modified white model cements	138
7	In vitro study of model cement and MTA containing PoP.....	144
7.1	Periosteal cultures containing modified grey model cements.....	144
7.2	Osteoblastic cultures containing modified grey model cements.....	146
7.3	Neonatal BMSC cultures containing modified grey model cements.....	147
7.3.1	Cells seeded onto cement in 4 ml of culture medium.....	147
7.3.2	Cells seeded onto cements in 50 µl of culture medium.....	148
7.3.3	Cements placed into established cell cultures	150
7.4	Adult BMSC cultures containing modified model cements and MTA	151
7.4.1	Cells seeded into 4 ml of culture medium	151
7.4.2	Cells seeded into small volumes (50 µl) of culture medium	155
7.4.3	Cement samples placed onto established adult BMSC cultures.....	156
7.4.4	Cell attachment to cement samples only	158
7.4.5	Cell attachment to cement paste only	160
7.4.6	Cell cultures containing cements pre-washed in various solutions	162
7.4.7	Cultures containing commercial MTA	164
8	Sealing ability of model cements and MTA with additives	170
8.1	Development of a tooth model: dye and ink permeation studies.....	170
8.1.1	Measurements taken using a grid	170
8.1.2	User dependency of the method established for determining dye permeation.....	174
8.2	Effect of PLR on dye and ink permeation into cements	176
8.2.1	0.5 % Crystal violet permeation	176
8.2.2	2 % Crystal violet permeation	177
8.2.3	0.5 % Safranin O permeation	179
8.2.4	2 % Safranin O permeation	180
8.2.5	India ink permeation	182
8.3	Effect of PoP addition on dye and ink permeation through cements.....	182
8.3.1	Crystal violet permeation.....	182
8.3.2	Safranin O permeation.....	183
8.3.3	India ink permeation	184
8.4	Sealing ability of model cements and commercial MTA containing PoP: tooth model	185

9	DISCUSSION.....	187
9.1	Characterisation and generation of a model system for MTA.....	187
9.1.1	Selecting cements for use as components of the model system	187
9.1.2	Selecting the powder to liquid ratio (PLR) of the model system	188
9.1.3	Selecting the type of bismuth oxide for use as a component of the model system	188
9.1.4	Changing the methodology of sample production.....	189
9.2	Effect of calcium sulphate additions on the model system.....	189
9.2.1	Selecting calcium sulphates.....	189
9.2.2	5-30 wt% calcium sulphate additions to model cement (white and grey).....	190
9.2.3	5 wt% PoP + 5 wt% CaSO ₄ additions	192
9.3	Effect of PoP on the setting and mechanical properties of MTA	192
9.4	Sterilisation of powders and set cements	196
9.5	Long term stability and solubility of PC containing PoP	197
9.5.1	Water storage.....	197
9.5.2	Culture medium storage	200
9.6	Effect of modified model cements and MTA on different cell cultures	202
9.6.1	Cements in periosteal and osteoblast cultures	202
9.6.2	Effect of model cements on neonatal and adult BMSC cultures.....	204
9.6.3	Comparing the cell types used for the study	210
9.6.4	Effect of modified MTA on adult BMSC cultures	211
9.7	Sealing capabilities of modified MTA and the MTA model cement.....	215
9.7.1	Development of a tooth model: dye permeation studies	215
9.7.2	Effect of PLR on dye permeation into cements.....	217
9.7.3	Effect of PoP addition on dye permeation into cements	219
9.7.4	The sealing ability of modified MTA using dye leakage	221
10	CONCLUSIONS	224
10.1	Effect of setting modifiers on mechanical properties of MTA and model cement...	224
10.2	Ageing of MTA and model cement containing PoP	225
10.3	In vitro effect of PoP on MTA and model cement.....	225
10.4	Sealing capability of MTA and model cement with PoP	226
11	FURTHER WORK.....	228
11.1	Effects of blood admixing with PoP-modified MTA on ageing	228
11.2	In vitro effect of MTA with PoP additions on cytotoxicity and cell attachment	228
11.3	Sealing capabilities of MTA with PoP using different methods.....	229
11.4	Antimicrobial effect of MTA containing PoP.....	231
11.4.1	Incorporating antibiotics into PoP-modified MTA root filling	231
11.5	The addition of other setting modifiers to MTA	232
	REFERENCES	234

APPENDIX I	259
1 Selection of bismuth oxide component to model system	259
2 Addition of PoP to MTA and model system	259
3 Long term stability and solubility of MTA-like cements containing PoP.....	260
3.1 Grey model cements with or without PoP	260
3.2 White model cements with or without PoP	262
4 <i>In vitro</i> Effect of model cements containing PoP on adult BMSC cultures	264
4.1 Cultures containing 24 h-set cement paste.....	264
4.2 Cultures containing modified MTA and model cements	265
5 Sealing Ability of MTA and model cements containing PoP	266
5.1 Effect of varying the PLR of model cement on Dye permeation	266

LIST OF FIGURES

Figure 1: An adapted diagram to show the structure of a tooth (a longitudinal cut section) [Atkinson <i>et al</i> , 1992].....	23
Figure 2: Setting of cement paste [National Research Council, 1997]. Arrows A: Pointing to the final products, CSH and Ca(OH)_2 , represents an important set of reactions. Arrow B: Points to the final product, C_4AF , produced through the hydration of C_3A	9
Figure 3: A schematic diagram to represent the preparation of the calvaria and removal of parietal bones to attain osteoblastic and periosteal cultures. The calvaria were trimmed as represented by (a), and then parietal bones separated from occipal and frontal bones (b). The parietal bones were cut along the parietal side of the saggital suture (c) and after the removal of the periosteum, cut into squares to attain primary osteoblastic cultures (d).	64
Figure 4: Images representing how dye or ink permeation through cement was measured using two methods, the first (1) representing the method used initially by taking randomly spaced measurements. For reliability of the measurements, an improved method (second method) was developed that involved taking measurements at set points. These points were determined by the presence of gridlines placed superficially on the image as shown in the second image (2).	72
Figure 5: Image representing a tooth prepared to be root filled with cement.	75
Figure 6: Scanning electron microscope (SEM) photomicrographs of Portland cement powders. Mastercrete particles were smaller than Snowcrete, which in turn were smaller than Original Portland cement. MC had the most homogeneously-sized particles.	79
Figure 7: Compressive strength (CS) of PCs at various PLR. 100 wt% MC generally had a higher CS than other cement types and MC was the only cement which was workable at a PLR of 4.0 g/ml.	80
Figure 8: Relative porosity (RP) of Portland cements at various PLR. Little difference was observed for cements that had the same PLR. The RP generally decreased with increasing PLR. Error bars represent the approximate error of the method and calculations used to retrieve RP ($\pm 1\%$).	81
Figure 9: SEM photomicrographs of two types of Bi_2O_3 powder from the manufacturers (A) Acros Organics (Acros) and (B) Sigma Aldrich.	82
Figure 10: Scanning electron micrographs of cements containing Bi_2O_3 from different manufacturers. The macro and microstructures appeared to be very similar regardless of the type of Bi_2O_3 added to the cements.....	83

Figure 11: CS of the model cement set for various lengths of time before water storage. CS decreased when the model cement was immersed in water after setting for 7 days when compared with cements set for shorter time periods. Error bars represent the standard deviations for the mean data plotted in the histogram..... 84

Figure 12: RP of model cements that were set for different time periods before water storage. Error bars represent the total error of the method and calculations used to retrieve RP ($\pm 1\%$). RP increased when cements were stored for 7 days but remained low with shorter setting times before water immersion. 84

Figure 13: SEM photomicrographs of the outer surface of cement samples. Samples were placed into water at different time periods. Image A. Cement placed into water after 6 hours of setting; Image B. after 1 day of setting; Image C. after 3 days of setting and Image D. after 7 days of setting. 85

Figure 14: SEM photomicrograph of Plaster of Paris (PoP) powder showing heterogeneously sized particles. 87

Figure 15: SEM photomicrographs of additive powders. Large differences were found between calcium sulphates, particularly CaSO_4 from different manufacturers as larger particles were observed in Acros Organics CaSO_4 when compared with particles in Sigma Aldrich CaSO_4 88

Figure 16: Initial setting times of the model cement containing the different calcium sulphates at various wt%. Initial setting times decreased considerably with increased additions of calcium sulphates with the most dramatic decrease in setting time observed when adding PoP or CaSO_4 (Sigma) to cement. Error bars represent the total error of the method (± 5 min)..... 89

Figure 17: CS of the model cement containing various calcium sulphate additives. PoP-containing cements always had a higher CS than the control. Large additions of calcium sulphates to MC generally led to a decrease in CS. Error bars represent the standard deviations of the mean results plotted on the graph..... 90

Figure 18: RP of the model cement containing various calcium sulphate additives. Gyp-containing cements showed the highest RP for all levels of additions. Small additions (5 wt%) of calcium sulphates PoP and CaSO_4 (Sigma and Acros) had little effect on the RP of cement. Large additions of these calcium sulphates increased the RP of cements. Error bars represent the total error of the method and calculations used to retrieve RP ($\pm 1\%$)...... 92

Figure 19: Initial setting times for the model cement containing calcium sulphates. Model cements with no additions of calcium sulphates and Gyp-containing cements did not set in the investigated time period. Increasing the amount of calcium sulphates additions, CaSO_4 and PoP decreased the setting times the most. Error bars represent the total error of the method (± 5 min). 94

Figure 20: CS of model cements containing 5 to 30 wt% additions of calcium sulphates. 5 and 10 wt% PoP and CaSO ₄ additions increased the CS of cements; however, increased addition (20 and 30 wt%) of PoP and CaSO ₄ decreases CS. Error bars represent the standard deviations for each mean value plotted.	96
Figure 21: RP of model cements containing additives of various weight percentages (wt%). Small increases in RP were evident with the increasing PoP and CaSO ₄ content. Large additions of calcium sulphates generally generated the highest RP in MC. Error bars represent the total error of the method and calculations used to retrieve RP ($\pm 1\%$).	97
Figure 22: SEM photomicrographs of the fracture surface of A. the model cement and B. cement containing 5 wt% PoP. The structure of cement did not appear to differ with the addition of low concentrations of PoP to cement.	98
Figure 23: SEM photomicrographs of the fracture surface of cement samples containing A. 20 wt% PoP and B. 20 wt% Gyp. Gypsum particles (indicated by arrows) were seen in cements containing 20 wt% PoP and 20 wt% Gyp.	99
Figure 24: SEM photomicrographs of the fracture surfaces of the model cement (A) and cement containing 20 wt% CaSO ₄ (B). More microcracks were evident in 20 wt% CaSO ₄ -containing MC when compared with the control.	100
Figure 25: SEM photomicrograph of the fracture surface of 5 wt% PoP + 5 wt% CaSO ₄ -containing cement, showing that large voids were present in the cement structure.	100
Figure 26: SEM photomicrographs of the outer surface of cement samples containing: A. 5 wt% PoP and B. 20 wt% PoP. Large needle like crystals were present in 5 wt% PoP-containing MC, however, none were visible in 20 wt% PoP-containing cements.	101
Figure 27: SEM photomicrographs of the outer surface of A: the model cement and B: 20 wt% CaSO ₄ -containing cements. Cracks were present in cements containing 20 wt% CaSO ₄ , which are indicated by the arrows.	102
Figure 28: SEM photomicrographs of the outer surface of cement samples containing A: 5 wt% Gyp-containing cement and B: 20 wt% Gyp-containing cement. Pores (indicated by arrows) and a poorly-connected structure were seen in the SEM micrograph B of cements containing 20 wt% Gyp.	102
Figure 29: Initial setting times of the SC-model cement containing PoP. Cements without PoP and cements containing Gyp did not set in the investigated time period of 6 h. Error bars for setting time measurements were ± 5 min, which was the estimated error of the method.	103
Figure 30: CS of the white SC-model cement with or without PoP additions. Cements with PoP had the highest CS compared with model cements and cements with Gyp additions. Standard deviations are present as error bars on the graph for the mean points plotted.	104

Figure 31: RP of the white SC-model cement containing PoP. Model cements with 5 and 10 wt% PoP additions had the lowest RP compared with the white SC-model cement with no additions and cements with Gyp additions. The approximate error of the method was $\pm 1\%$ 105

Figure 32: The effect of PoP additions on the initial setting time of the model system and MTA. Cements containing no addition of PoP set in over 4 h. MTA containing PoP was similar to the model cement containing PoP which was considerably reduced when compared with cement without PoP. The error bars represent the minimum and maximum error attached to the experimental method was ± 5 min..... 106

Figure 33: CS of MTA, the model system and cements modified with PoP. Similar CS was observed for MTA and the model system, however when cements were modified with PoP, the CS decreased the most for the model cement when compared with the CS of modified MTA. Standard deviations (\pm) were placed as error bars for each of the mean points plotted..... 107

Figure 34: RP of MC and MTA cements containing additives of 10 wt% PoP. The addition of PoP increased the RP in cements. Error bars represent the total error of the method and calculations used to retrieve RP ($\pm 1\%$). 108

Figure 35: SEM micrographs of the outer surface of the model cement (A) and MTA (B) control samples, showing that MTA control had a more compact structure when compared with the model cement..... 109

Figure 36: SEM photomicrographs comparing the differences between the outer surface (1) and fracture surfaces (2) of the model cement (A) and MTA control (B) samples. MTA had small, needle-like particles present on the outer surface (B1) which differed to the model cement which had mainly large particles on the outer surface (A1). Microcracks were seen in the SEM photomicrograph of the outer (B1) and fracture surfaces (B2) of MTA. 110

Figure 37: SEM photomicrographs showing the effect of 10 wt% PoP addition on the outer (1) and fracture surfaces (2) of the model cement (A) and MTA (B). The white arrows in images A2 and B2 point to the large crystals present within modified model cement and modified MTA. A similar fracture surface was apparent for model cement and MTA that both contained 10 wt% PoP (A2 and B2) which contrasted the appearance of the outer surfaces of these cements that contained 10 wt% PoP (A1 and B1)..... 111

Figure 38: XRD patterns of cements containing various amounts of calcium sulphates. Residual gypsum was detected in 20 wt% PoP-containing cements. All cements showed some degree of ettringite formation and the presence of calcium hydroxide. 112

Figure 39: X-ray diffraction patterns for MTA, the model system and cements containing 10 wt% PoP. MTA and the model system had the same crystalline phases present, both showing additional gypsum and ettringite phases in the cements with PoP addition..... 113

Figure 40: Graph showing the molar concentration of calcium in water (from calcium sulphates) with time. PoP had a similar kinetic solubility to CaSO_4 which was more soluble than Gyp. Approximate error of the method was $\pm 20\%$ 115

Figure 41: CS of model cements with or without PoP additions that were sterilised using different treatments. CS generally decreased when cements were sterilised when compared with cements that were not sterilised. Standard deviations of mean values are present on the graph as error bars. 116

Figure 42: Bar chart to represent RP of cements with or without the addition of PoP which were sterilised using different methods. Error bars represent the approximate error of the method and calculations used to retrieve RP ($\pm 1\%$). RP were higher for cements that had been autoclaved or had treated with dry heat than those which were not sterilised. 117

Figure 43: CS of grey model system with or without additions of PoP, stored in distilled water for up to 12 months. Error bars on the graph represent the standard deviations for mean values that were plotted. Strength declined considerably with length of storage time for 30 wt% PoP-containing cements. 119

Figure 44: RP of grey model cements with or without added PoP which were stored in distilled water for up to 12 months. Error bars represent the approximate error of the method and calculations used to retrieve RP ($\pm 1\%$). RP were observed to increase noticeably when the model cements containing 30 wt% PoP were stored with time. 120

Figure 45: CS of white model cement with or without PoP additions at various time points. Error bars on the graph represent the standard deviations for mean values that were plotted. 10 wt% PoP additions to the white model cement increased slightly with increased storage time in distilled water. 121

Figure 46: RP of the white model cement and cements containing PoP additions stored in distilled water. Error bars represent the approximate error of the method and calculations used to retrieve RP ($\pm 1\%$). Cements with PoP additions tended to have higher RP when compared with the white model cement only. Cements with PoP additions had increased RP with increased storage time. 122

Figure 47: Bar chart to represent CS of the grey model cement with or without PoP and set for different lengths of time in culture medium. Error bars on the graph represent the standard deviations for mean values that were plotted. Cements with or without 30 wt% PoP additions had similar CS at 10, 30 days and 3 months. However, the highest CS were observed for cements containing 10 wt% PoP additions. 124

Figure 48: Bar chart to represent the RP of cements stored in culture medium with or without PoP additions. Error bars represent the approximate error of the method combined with the error attached to calculating the RP ($\pm 1\%$). Grey model cements without PoP and cement containing 10 wt% PoP had RP that generally decreased with time, though the cements containing PoP had higher RP when compared with cements without PoP. 125

Figure 49: CS of cements with or without PoP that have been stored in culture medium for up to 9 months. Error bars on the graph represent the standard deviations for mean values that were plotted. Culture medium storage of 10 wt% PoP-containing cement had higher CS with storage time when compared with other cements stored in culture medium. 126

Figure 50: RP of cements with or without PoP additions that had been stored in culture medium for up to 9 months. Error bars represent the approximate error of the method and calculations used to retrieve RP ($\pm 1\%$). RP varied according to composition, however, the white SC model cements without PoP had the lowest RP when compared with cements containing 10 or 30 wt% PoP..... 127

Figure 51: The release of calcium ions (M) from model cements with or without PoP additions into water (which cements were stored within) with time (weeks). The estimated approximate error of the method used to obtain the data plotted was $\pm 20\%$. Approximate errors were not plotted on the graph as error bars so that plot points may be seen more clearly. There were more calcium ions released from cements with or without PoP additions into water, when measured in week 4..... 129

Figure 52: SEM photomicrographs of the outer and fracture surfaces of the grey model cement with or without PoP additions stored in water for 10 days. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images to the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. More pores were apparent in the outer surface of 30 wt% PoP-containing grey model cement compared with unmodified grey model cement and 10 wt% PoP containing grey cement. 131

Figure 53: SEM photomicrographs of grey model cements stored in water for 9 months. Images to the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images to the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. The fracture surfaces of PoP-modified grey cements appeared to be denser than unmodified model cements..... 132

Figure 54: SEM photomicrographs of the outer (A) and fracture (B) surfaces of MC model cements stored in water for 9 months. Large cement plates were evident on the fracture surface at x 2000 magnification, however, small needle-like particles were observed on the outer surface of the model cement..... 133

Figure 55: SEM photomicrographs of the outer and fracture surfaces of the MC model cement with or without PoP additions stored in culture medium for 10 days. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Cement structures were less compact when PoP content was increased..... 135

Figure 56: SEM photomicrographs of the outer and fracture surfaces of the MC model cement with or without PoP additions stored in culture medium for 9 months. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Density decreased with increased PoP content to grey model cements and cracks were apparent in PoP-modified grey cements. 137

Figure 57: SEM photomicrographs of the outer and fracture surfaces of the white SC model cement with or without PoP additions stored in water for 10 days. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Outer and fracture surfaces were similar in appearance regardless of cement composition. 139

Figure 58: SEM photomicrographs of the outer and fracture surfaces of the white SC model cement with or without PoP additions stored in water for 9 months. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Fracture surfaces were less porous compared with outer surfaces of unmodified and PoP-modified cements. 140

Figure 59: SEM photomicrographs of the outer and fracture surfaces of PoP-modified white SC model cement stored in culture medium for 10 days. Images on the left (A, C and E) represent the outer surfaces of unmodified and 10 or 30 wt% PoP-modified model cement, respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. More pores and gaps were present in the fracture and outer surfaces of 30 wt% PoP-modified cements. 142

Figure 60: SEM photomicrographs of the outer and fracture surfaces of the white SC model cement with or without PoP additions stored in culture medium for 9 months. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Fracture surfaces were denser than the outer surfaces of cements, which consisted of rounded clusters of cement particles rather than cement plates. 143

Figure 61: The cell densities (total from culture well/ culture well and cement) of periosteal cultures containing cement samples with or without PoP with time (14days) No cells attached to cements with or without PoP. Similar cell densities were observed for cultures containing model cements and cements with PoP additions, but significantly higher for control cultures ($p<0.05$). Error bars represent the standard deviation of the mean values. 145

Figure 62: Density of viable osteoblasts attached to cell culture wells (and cells attached to cement) with or without model cements that had or had not been modified with PoP. Cultures containing cements modified with or without 30 wt% PoP had lower cell counts when compared with cultures containing just primary osteoblasts. 146

Figure 63: Total number of viable neonatal BMSCs (seeded directly onto cements in 4 ml of medium) attached to cell culture wells (and cells attached to cement) with or without model cements and PoP modified model cement. Control neonatal BMSC cultures had significantly higher ($p<0.05$) cell densities compared with cultures containing model cements with or without PoP, by the end of the experimental period of 14days. Cell densities for cultures containing cements were similar throughout the experimental period. 148

Figure 64: Total number of viable neonatal BMSCs (seeded directly onto cements in 50 μ l of medium and cultured for an hour) counted in cultures that contain cements with different compositions after being cultured for up to 10 days. The graph showed that cultures containing model cements with or without 30 wt% PoP had significantly lower densities compared with control cultures that consisted of neonatal BMSCs only..... 149

Figure 65: Cell densities of viable neonatal BMSCs counted after 3 days in established cultures that contained the model cement with or without PoP additions cultured. The dashed line represents the cell density seeded into culture wells at the beginning of the experiment. The combined density of cells attached to culture wells and cements was recorded. Cultures that contained PoP-modified or unmodified model cements had significantly lower cell densities recorded compared with control cultures. 150

Figure 66: The density of viable adult BMSCs (seeded directly onto cements in total volume 4 ml of medium) in cultures that contain cements with or without PoP additions. Control cultures that consisted of adult BMSCs only had significantly higher cell densities compared with cultures with model cements containing 30 wt% PoP, which had slightly higher cell densities compared with model cement cultures. Error bars on the graph represent the standard deviations of the mean values plotted. 152

Figure 67: SEM photomicrographs of BMSCs in cultures on glass cover slips (A) and cultures with model cements without PoP additions (B). Cells appeared poorly attached to model cements since no filopodia were apparent when compared with BMSCs from cultures that consisted of these cells only after 3 days of culturing. 153

Figure 68: SEM photomicrograph of BMSCs in cultures containing model cements with 30 wt% PoP addition. Cells appeared poorly attached since few filopodia were present and appeared to bridge between cement particles where there are pores in the cement structure..... 154

Figure 69: Phase contrast images of ‘live-dead’ stained cultures with (image B) or without model cements (image A). A few damaged adult BMSCs were observed to be attached to culture wells (indicated by red nuclei, see red arrow) near to model cements (dark black area in top left hand corner of image B) that did not contain additions, indicated by the red colour of cells..... 154

Figure 70: Total number of viable adult BMSCs (seeded directly onto cements in 50 μ l of culture medium and cultured for one hour) counted in cultures that contain cements with different compositions after being cultured for up to 10 days. 155

Figure 71: Density of viable neonatal BMSCs counted after 3 days in established cultures that contained the model cement with or without PoP additions cultured. The dashed line represents the cell density seeded into culture wells at the beginning of the experiment. Cell densities increased for all cultures but were higher for control cultures that consisted of adult BMSCs only compared with cultures containing cements..... 156

Figure 72: Phase contrast images of cultures with or without cement samples to show the density of cells in these cultures with time (3 days). Cell detachment was observed for cultures containing cements, which increased with time of culturing. Cells in control cultures appeared to proliferate with increased culture time since more cells were observed at 1 day compared with 3 days. 157

Figure 73: Cell attachment to cements and culture wells only after 3 days. Counts for the number of viable and non-viable cells were made as shown in the figure. Very few cells attached to grey model cement containing 30 wt% PoP. Error bars represent the standard deviations of mean values. 158

Figure 74: Bar chart to represent the number of viable and non-viable dead/damage cells in the medium of cultures containing cements with or without PoP after 3 days. Error bars represent the standard deviations of mean values. No viable cells were counted for cultures containing modified white model cements and no non-viable cells were present in culture medium from modified grey model cement-containing cultures. 159

Figure 75: Bar chart to represent the pH of culture medium from cultures that contained the white or grey model cement with or without 30 wt% PoP additions after 1 day of culturing. The pH of culture medium increased with the presence of cements in cultures. Error bars represent the standard deviations of mean values. 160

Figure 76: Bar chart to represent the density of cells attached to culture wells (for BMSC culture only) and cement pastes containing 30 wt% PoP. Error bars represent the standard deviations of mean values. Few cells attached to cement pastes compared with control cultures consisting of adult BMSCs only. 161

Figure 77: Bar chart to represent the total number of non-adherent, viable cells present in medium from cultures containing cement paste. Error bars represent the standard deviations of mean values. Few cells were present in the culture medium extracted from cultures that contained cement pastes compared with control cultures of adult BMSCs. 162

Figure 78: Bar chart to represent the density of viable adult BMSCs in cultures containing the model cement that were or were not washed with different solutions (cells attached to culture plastic). Cells only attached to cements that were washed in PBS. Error bars represent the standard deviations of mean values. 163

Figure 79: Bar chart to represent the viability of adult BMSCs attached to culture plastic in cultures that contained the model cement washed with different solutions compared with cells attached to culture wells of cultures containing unwashed cements. Error bars represent the standard deviations of mean values. 164

Figure 80: The density of viable BMSCs attached to culture plastic and cements in cultures containing MTA and the model cement with or without 10 wt% PoP. Error bars represent the standard deviations of mean values. Significantly higher cell densities were recorded for MTA-containing cultures at 14days compared with model cement-containing cultures. 165

Figure 81: SEM photomicrographs of cells grown on glass slides in close proximity to cements. Images represent Adult BMSC cultures containing (A) MTA, (B) MTA modified with 10 wt% PoP, (C) the model cement and (D) the model cement containing 10 wt% PoP. Zones of cell growth inhibition were observed in cultures containing cements.	166
Figure 82: SEM photomicrographs of cells that had grown on glass slides in close proximity to MTA, indicated by white arrows and layers of cells labelled. A growth inhibition zone for BMSCs was observed surrounding MTA.....	167
Figure 83: SEM photomicrographs of BMSCs that had grown on glass slides in culture wells. BMSCs were well attached and spread on the glass slides in culture wells, forming sheets of cells after 14days in cultures.....	168
Figure 84: SEM photomicrographs of MTA (A) and MTA with 10 wt% PoP additions (B) that had been in cultures containing BMSCs for 14days. No BMSCs had colonised MTA or MTA with PoP.....	168
Figure 85: SEM photomicrographs of model cements without PoP (A) and 10 wt% PoP-containing model cements (B) that had been in cultures containing BMSCs for 14days. No BMSCs were found to have colonised on model cements with or without PoP and possible cell debris was observed on PoP-containing cements indicated by the arrows.	169
Figure 86: The permeation of various dyes at varying concentrations through model cements which were set for 1 days prior to being placed into dye for 1 days. Standard deviations were represented as error bars on the graph. 2 % Rhodamine B permeated through model cements the most when compared with other dyes at different concentrations.	170
Figure 87: Bar chart to represent the permeation various dyes at varying concentrations through model cements which were set for 1 days prior to being placed into dye for 3 days. Standard deviations were represented as error bars on the graph. Regardless of the concentration, Rhodamine B permeated through model cements the most when compared with other dyes at different concentrations.	171
Figure 88: Bar chart to represent the permeation various dyes at varying concentrations through model cements which were set for 1 days prior to being placed into dye for 7 days. Standard deviations were represented as error bars on the graph. Rhodamine B permeated through model cements the most when compared with other dyes at different concentrations.	172
Figure 89: Depths of dye permeation through model cements with different dyes and concentrations which were stored for 3 days prior to being placed into dye solutions for 3 days. Standard deviations were represented as error bars on the graph. 2 % Methylene blue permeated through model cements the least when compared with other dyes at varying concentrations....	173
Figure 90: Bar chart to represent the depths of dye permeation through model cements which were stored for 7 days prior to being placed into different dyes and concentrations for 3 days. Standard deviations were represented as error bars on the graph. 1 % Safranin O permeated through model cements the least when compared with other dyes at varying concentrations....	174

Figure 91: Bar chart to represent dye permeation through model cements, when different dyes and concentrations were used and the measurement of dye permeation was taken by two different users. Standard deviations were represented as error bars on the graph. Similar depths of 1 % Crystal violet were measured by the two users in the study..... 175

Figure 92: Depths of dye permeation (0.5 % Crystal violet) through model cements with different PLR which were stored for 1, 10 and 30 days prior to being placed into dye. Standard deviations were represented as error bars on the graph. Cements with a PLR of 2.5 g/ml had significantly more dye permeation after 1 days of setting compared with other PLR set for 10 and 30 days before dye immersion. 177

Figure 93: Depths of dye permeation through model cements with different PLR which were stored for 1, 10 and 30 days prior to being placed into 2 % Crystal violet solutions. Standard deviations were represented as error bars on the graph. Cements with a PLR of 2.5 g/ml had the highest values recorded for dye permeation when compared with other PLR stored for the same length of time before being placed into dye. 178

Figure 94: The depth of Safranin O (0.5 %) permeation through model cements with different PLR. Standard deviations were represented as error bars on the graph. More 0.5 % Safranin O permeated through model cement with a PLR of 2.5 g/ml which had been stored for 1 day prior to immersion in dye. 180

Figure 95: Bar chart to represent the depth of Safranin O (2 %) permeation through model cements with different PLR. Standard deviations were represented as error bars on the graph. Model cement with a PLR of 2.5 g/ml had the greatest depth of dye permeation when stored for 1 and 10 days prior to immersion in 2 % Safranin O. 181

Figure 96: Crystal violet dye permeation through model cements only and model cements consisting of 10 and 30 wt% PoP. Standard deviations were represented as error bars on the graph. Cements stored for only 1 day prior to Crystal violet storage had the greatest dye permeation recorded when compared with 10 and 30 days of cement storage before dye immersion. 183

Figure 97: Bar chart to represent the depth of Safranin O dye permeation through cements with or without PoP additions and stored for varying lengths of time before immersion in Safranin O dye. Standard deviations were represented as error bars on the graph. Safranin O dye permeated through model cements the least when compared with 10 and 30 wt% PoP additions which had been permeated entirely..... 184

Figure 98: Bar chart to represent the dye permeation and penetration of 5 % Crystal violet into root filling cements. Standard deviations were represented as error bars for the mean values recorded on the graph. MTA containing 10 wt% PoP had the greatest amount to dye permeate through the root filling, however, did not allow dye to penetrate the cement much at all like model cement root fillings with or without PoP additions. 185

1 INTRODUCTION

1.1 *Structure of the tooth*

The tooth is composed of three mineralised tissues these being enamel, dentine and cementum (see figure 1) which surround loose connective tissue in the form of the dental pulp [Avery *et al.*, 2002].

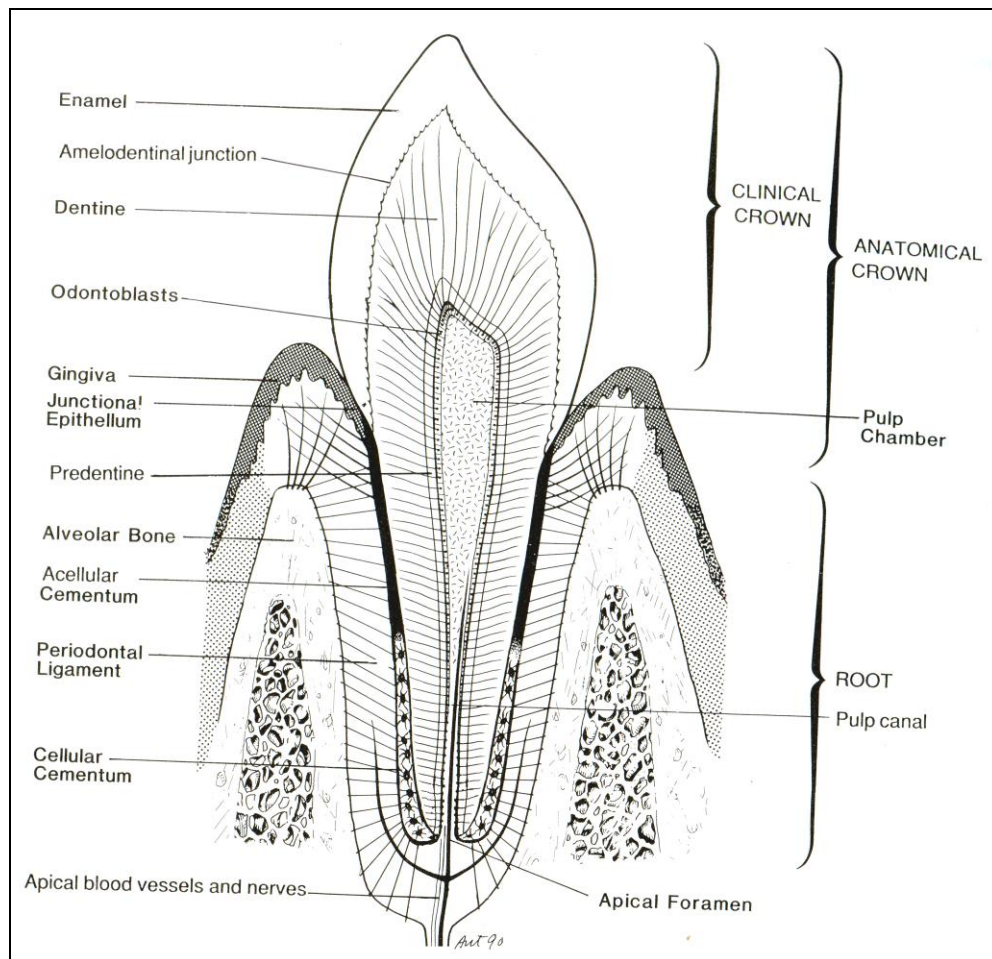


Figure 1: An adapted diagram to show the structure of a tooth (a longitudinal cut section) [Atkinson *et al*, 1992].

INTRODUCTION

The enamel is a highly mineralised protective layer which is secreted by ameloblasts present within this part of the tooth [Atkinson *et al*, 1992]. Enamel and dentine connect at the dentinoenamel junction, or more commonly known as the amelodentinal junction.

Dentine is comprised of organic and inorganic portions. The organic part of dentine consists of four layers which are listed in descending order towards the pulp: dentine, predentine, a layer of odontoblasts (responsible for the deposition of dentine for maintenance and repair) and dental pulp cells. The inorganic component consists of hydroxyapatite crystals and inorganic dentine [Avery *et al*, 2002]. Root filling materials would be likely to come into contact with dentine should the dental pulp be fully removed from the tooth.

Cementum is an amorphous, calcified tissue which lies hidden beneath the gingiva that meets enamel at the cement-enamel junction. Cementoblasts and cementocytes are responsible for the maintenance and production of cementum and are present in cellular cementum [Avery *et al*, 2002]. These function similarly to bone cells in that the generation of a mineralised tissue is achieved by cementoblasts in cementum and osteoblasts in bone, but this tissue is also resorbed by cementocytes in cementum and osteocytes in bone [Avery *et al*, 2002].

The pulp is a soft tissue compared with enamel and dentine that consists of delicate supporting/connective mesenchymal tissue, which includes stellate fibroblasts, ground substance, fine collagen and reticulin fibres. Odontoblasts are present in a layer surrounding the dentine that is adjacent to the pulp, which may be referred to as the pulp-dentine complex. A network of capillaries and arterioles enter the pulp at the periodontal membrane located at the apex of the tooth, so do myelinated and non-myelinated nerve fibres [Avery *et al*, 2002].

1.2 *Root canal treatment: the need for root canal filling materials*

The pulpal tissues of the tooth may become inflamed or injured as a result of bacteria entering the root canal, mechanical or chemical irritants being exposed to the tissues or as a result of damage to the blood supply of the tooth. Necrosis of the pulp tissue could occur that could give rise to the development of an infection in the root canal. The damaged and infected tissue must therefore be removed to sustain the health and functionality of the tooth [Lumley *et al*, 2006]. To prevent the spread of bacteria, root filling materials are inserted to replace the space once occupied by damage and necrotic pulpal tissues.

1.3 *Limitations of root filling materials: introduction of mineral trioxide aggregate*

Root filling materials are expected to provide a hermetic seal of the root canal in order to prevent bacteria from entering surrounding tissues, thus preventing spreading infection which could result in the requirement for further root canal therapy or the possibility of either tooth loss or even bone loss [Chng *et al*, 2005, Islam *et al*, 2006]. It would be advantageous should the seal be provided shortly after placement of the root filling material because a hermetic seal would be likely to be ensured, meaning the likelihood of bacteria entering the root canal would be considerably reduced. Another advantage of an immediate seal to the root could mean that patients would be less likely to require further post operative checks which would have a positive impact by saving surgeries time and money as a result. Improving the setting time of root filling materials and thus providing an immediate hermetic seal would therefore have positive implications on patients and dental surgeries which may not have already been explored. A

INTRODUCTION

suitable material for endodontic treatment should also demonstrate biocompatibility (and possibly bioactivity), the ability to adhere to the tooth structure, maintain dimensional stability, be insoluble in tissue fluids, be non-resorbable [Danesh *et al*, 2006] and should have adequate radiopacity to be seen amongst the surrounding tissue in radiographic assessment post-placement [Chong *et al*, 2005, Roberts *et al*, 2008].

Many root filling materials have been employed as part of root canal treatment and all of these materials have limitations to their use. Amalgam has been used for several decades, not only for endodontic treatments but also for other dental applications such as being commonly used as a restorative material. One of the limitations of the use of amalgam is the higher level of cytotoxicity that the material has been reported to cause when compared with other root filling materials such as Gutta Percha, Glass ionomer cements, Super EBA and composite resins [Vasudev *et al*, 2003].

Mineral trioxide aggregate, MTA, was introduced as an endodontic material for pulp capping, treating internal and external root-resorption, perforation repairs and root fillings [Torabinejad *et al*, 1999, Walker *et al*, 2006]. This was due to its reported advantageous properties over other root filling materials, including its excellent sealing ability, biocompatibility, durability and good handling properties [Chng *et al*, 2005, Islam *et al*, 2006]. One limitation to the use of MTA is the long setting time of the material [Torabinejad *et al*, 1993] which will be discussed further in the following sections to come.

MTA is a modified Portland cement (PC) which contains bismuth oxide, Bi_2O_3 , added for radiopacity [Torabinejad *et al*, 1993]. PC was considered an inexpensive alternative to MTA because there is little difference in the composition of these cements, apart from the presence of Bi_2O_3 in MTA. Both MTA and PC share the same chemical composition, sharing 14 chemical elements (observed using energy dispersive analysis by X-ray) [Camillieri *et al*, 2006a].

INTRODUCTION

There are three commercially available MTA which are all based on PC as previously discussed: ProRoot MTA (grey and white), MTA Angelus (grey) and MTA Bio (tooth-coloured). Set commercial MTA consists of ~50-70% calcium oxide and ~15-25 % silicon dioxide that contribute to up to 80% of the cement and ~20% Bi_2O_3 in MTA [Camillieri *et al*, 2006a]. White commercial MTA (introduced since it was more aesthetically pleasing [Asgary *et al*, 2005]); contains calcium, silicon, bismuth and oxygen, and grey MTA contains the same as those just listed but also contains iron [Camillieri *et al*, 2005a, 2006a]. MTA contains low concentrations of carborundum (containing aluminium oxide (Al_2O_3)), periclase (containing magnesium oxide) and iron oxide (Fe_2O_3), which are chromophores that also give colour to the cement. [Dammaschke *et al*, 2005, Danesh *et al*, 2006]. Toxic heavy metals like manganese, strontium and copper were found in low levels in MTA [Dammaschke *et al*, 2005, Danesh *et al*, 2006], with lower levels present in white MTA when compared with grey MTA [Asgary *et al*, 2005].

In terms of particle size, MTA powder consists of fine hydrophilic particles that differ between white and grey MTA, due to the chemical composition [Torabinejad *et al*, 1993]. Grey MTA contains large particles [Asgary *et al*, 2005] whereas white MTA consists of heterogeneously sized particles, some similar in size to grey MTA particles and some smaller particles [Danesh *et al*, 2006]. PC has a smaller mean particle size in comparison with MTA [I. Islam *et al*, 2006, Sarkar *et al*, 2005, Abdullah *et al*, 2002].

1.4 ***Portland cement***

Portland cement, PC, (a component of MTA) is manufactured from lime (CaO), silica (SiO_2), alumina (Al_2O_3) and iron oxide (Fe_2O_3) which are crushed, ground and proportioned to the desired composition then heated to 1400-1600°C to form the clinker [Lea *et al*., 1970,

INTRODUCTION

National Research Council, 1997]. The clinker is ground with gypsum to form PC powder [Taylor, 1997].

The chemical composition of PC varies slightly in comparison with that of MTA because there is a large availability of many different types of PC, used mainly in the building industry for general construction use [Bye *et al.*, 1999, Taylor *et al.*, 1997]. The composition of the clinker (the end product of a PC kiln) in PC is adjusted and varied to produce the desired components of PC and thus the type required for application. PC powder contains four major anhydrous phases [Kosmatka *et al.*, 1995], including alite, C_3S , which is a tricalcium silicate ($3CaO \cdot SiO_2$), making up 50-70% of the PC clinker, belite, C_2S , a dicalcium silicate ($2CaO \cdot SiO_2$) constituting 15-30% of the PC clinker, aluminate (tricalcium aluminate, C_3A) and ferrite (calcium aluminoferrite, C_4AF) phases contributing 5-10% and 5-15 % of the clinker in PC respectively [Lea *et al.*, 1970, National Research Council, 1997, Taylor, 1997]. PC is typically grey in colour, however, may be produced with an increased ratio of Al_2O_3 to Fe_2O_3 for the production of white PC. Grey PC consists of approximately 67 % calcium oxide (CaO), 22 % silicon dioxide (SiO_2), 5 % Al_2O_3 , 3 % Fe_2O_3 and 3 % of other components such as gypsum, the latter required to control the setting time of PC [Taylor, 1997].

There are five main types of PC (recognised by the American Society for testing and materials, ASTM C 150), the first and second often characterised as ordinary Portland cements for general purpose use [Taylor, 1997]. Type I is often used as concrete whereas type II is usually required to produce foundations or footing due to the lower heat of hydration and a more moderate resistance to sulphate attack (i.e. contains less gypsum in the blend) [Taylor, 1997, American Society of Concrete Contractors, 2005]. The third cement type contains increased alite content and finer grinding of the clinker for rapid hardening of the cement paste and as a result high early strength but is less commonly used compared with cement types I and II. Cement types

INTRODUCTION

IV and V are much more rarely used in construction since cement type IV has been chemically modified to hydrate with a lower heat of hydration for the production of large structures such as dams and the fifth cement type possesses a high sulphate resistance for use in areas with high sulphate (i.e. high sulphate soils) [Taylor, 1997, American Society of Concrete Contractors, 2005].

1.5 *The hydration of Portland cement*

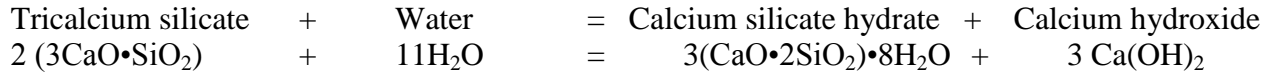
The four major anhydrous phases of unhydrated PC (and MTA), alite and belite (tri- and dicalcium silicates), ferrite (calcium aluminoferrite, C_4AF) and aluminate (tricalcium aluminate, C_3A), hydrate to form the two major final products, which are calcium oxide and amorphous calcium phases, these being calcium silicate hydrate (CSH; rigid or C-S-H; poorly crystalline material) and calcium hydroxide ($Ca(OH)_2$) [Lea *et al.*, 1970, Camilleri *et al.*, 2005]. The reaction proceeds to form a colloid gel that hardens on setting [Torabinejad *et al.*, 1993]. Many reactions occur at the same time during the hydration and setting of PC, during which by-products are formed, meaning that PC setting is extremely complex.

1.5.1 **Hydration of calcium silicate phases: alite and belite**

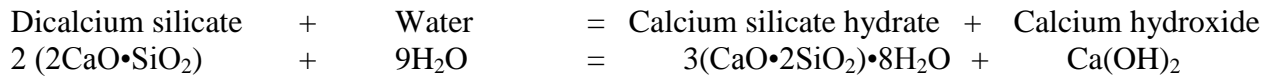
The hydration of the calcium silicates, alite (C_3S) and belite (C_2S), produces C-S-H (see equations below), the main component of cement hydration [Camilleri, 2007] and is responsible for the initial strengthening of the cement paste, otherwise known as hardening [Dammashke *et al.*, 2005].

INTRODUCTION

Hydration of alite (C_3S):



Hydration of belite (C_2S):



Ca(OH)_2 is produced as a product of C-S-H (a poorly crystalline calcium silicate hydrate) reactions (Reaction A: see Figure 2) [Ghosh, 2002]. Alite is one of the most important constituent phases for the development of early strength. Belite on the other hand reacts with water at a slower rate and contributes to the long term strength of the cement [Taylor, 1997]. Ca(OH)_2 buffers the pore solution to approximately pH 12.5 [National Research Council, 1997] and is thus responsible for the high alkalinity of both MTA and PC [Taylor, 1997].

1.5.2 Hydration of aluminate phases: tricalcium aluminate

Tricalcium aluminate, C_3A , is one of the most reactive species [National Research Council, 1997] and in the presence of other phases, reacts exothermically [Taylor, 1997] with water to form C_4AF crystals (Reaction B: see Figure 2) responsible for early hardening of the cement paste through rapid setting [Ghosh *et al*, 2002]. However, in the presence of gypsum, C_3A reacts with sulphate ions to form a protective layer, ettringite (calcium sulphotoaluminate, $C_6AS_3H_{32}$, a metastable phase, which later forms a monosulphate phase, C_4ASH_{12}) [Lea *et al.*, 1970, National Research Council, 1997], which prevents the rapid formation of C_4AF crystals [Kuzel *et al*, 1995], which is necessary in the building industry for applications where the strength of the cement needs to be improved by controlling the setting time [National Research

INTRODUCTION

Council, 1997]. Ettringite is later consumed to form monosulphate (see figure 2) [Camilleri, 2007] when further reacting with water due to an insufficient supply of sulphate ions [Taylor, 1997] which are required to complete the hydration of C_3A to C_4AF (Reaction B: see Figure 2) [Ghosh *et al*, 2002].

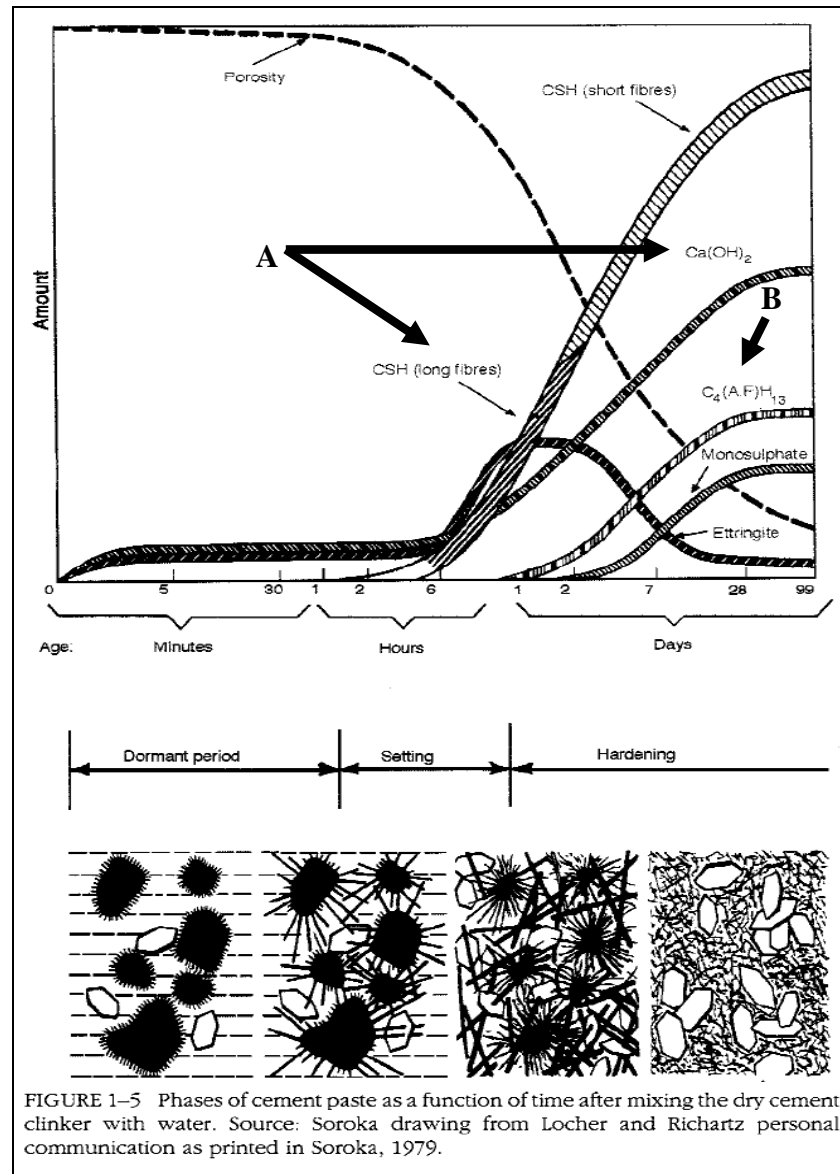


Figure 2: Setting of cement paste [National Research Council, 1997]. Arrows A: Pointing to the final products, CSH and $Ca(OH)_2$, represents an important set of reactions. Arrow B: Points to the final product, C_4AF , produced through the hydration of C_3A .

1.5.3 Final phases formed during setting

The final phases formed by the hydration of PC are calcium hydroxide [Asgary *et al.*, 2005], calcium-silicate-hydrate, ettringite (AFt phase) and monosulphates (AFm phase). Figure 2 illustrates the formation of hydration products as a consequence of alite, belite and aluminate phase consumption, which changes with time [Lea *et al.*, 1970, National Research Council, 1997].

1.5.4 Abnormal setting of Portland cement: 'Flash' setting

Without the presence of gypsum, PC 'flash' or 'quick' sets, which is describing a setting phenomenon where the early stages of setting are accelerated [Taylor, 1997]. C₃A phases hydrate due to the lack of ettringite formation (caused by insufficient supply of gypsum) surrounding C₃A particles (see section 1.1.3.2), releasing a lot of heat and causing the cement paste to stiffen [Ghosh *et al.*, 2002] to the point whereby plasticity cannot be regained with continual mixing, subsequently weakening the strength development of the cement due to the phases formed [Taylor, 1997]. As a consequence of an inadequate amount of gypsum present in the cement composition, monosulphate forms large plates (5-10µm in size) and the ettringite phases form small rod-shaped structures [Taylor, 1997, National Research Council, 1997].

1.5.5 Abnormal setting of Portland cement: 'False' setting

'False' setting of PC occurs in the presence of a non-fully hydrated form of calcium sulphate. This is an abnormal setting of PC, whereby the abundant supply of calcium sulphate in PC precipitates as gypsum with little or no heat evolution, thus causing stiffening of the cement paste. Initial plasticity can be restored on further mixing of the cement paste without the addition

INTRODUCTION

of more water [Lea *et al.*, 1970, Taylor, 1997, National Research Council, 1997] meaning that the cement may maintain a degree of workability if further mixed.

1.6 *Setting times of mineral trioxide aggregate (MTA) and Portland cement (PC)*

MTA and PC can set under physiological conditions [Islam *et al.*, 2006], which is important for application as a root filling material. However, the setting times of MTA and PC were longer than those of existing dental materials used in endodontics; amalgam, Super-EBA (a zinc oxide- based cement) and IRM (Intermediate restorative powder) have reported setting times of 4 ± 0.5 , 9 ± 0.5 and 6 ± 0.5 min respectively [Torabinejad *et al.*, 1995b]. The mean setting time of MTA was recorded using a Vicat needle as $2 \text{ h } 45 \pm 5 \text{ min}$ [Torabinejad *et al.*, 1995b] and others have suggested grey MTA set in $3 \text{ h } 22 \text{ min}$ (202 min) using a Vicat needle [Ber *et al.*, 2007] and $2 \text{ h } 55 \text{ min}$ (175 min) using a Gilmore needle [Islam *et al.*, 2006a]. White MTA sets in $41 \pm 1 \text{ min}$ [Gandolfi *et al.*, 2009] but in $2 \text{ h } 20 \text{ min}$ (140 min) [Islam *et al.*, 2006a] when investigated by others using the same method involving a Gilmore needle. PC was found to set within 3-4 h [Ber *et al.*, 2007] but it was reported that the setting time of PC with added bismuth oxide vary between 3 and over 6 h [Murphy *et al.*, 2008, O'Beirne *et al.*, 2008]. Commercial PC (applicable to both white and grey) were found to have potentially lower setting times than MTA (white and grey types) [Torabinejad *et al.*, 1995b].

The reactive species tricalcium aluminate, contained in PC and MTA, controls the setting time. It has been found that the sulphate component of gypsum shortens the setting time possibly by increasing the rate of reaction between the reactants in MTA [Dammasachke *et al.*, 2005].

INTRODUCTION

1.6.1 The effect of accelerants and modifications and the impact on CS

Setting accelerators, metal hydroxides and aluminates [Islam *et al*, 2006b] have been proposed to improve the setting time of MTA, through investigations using both PC and MTA itself. The compressive strength (CS) of MTA or PC is not directly important for the application of MTA or PC as an endodontic filling material as it is usually a non-load bearing material. However, CS can be used as an indicator for the mechanical stability and thus the potential longevity of the material. MTA or PC with high CS are likely to have fewer flaws and lower porosity in comparison with MTA or PC with low CS. Setting accelerators or modifications may cause the CS of MTA or PC employing additives to decrease which could compromise the sealing capacity of the material and thus its longevity [O'Beirne *et al*, 2008].

The addition of sodium hypochlorite gel and K-Y jelly both reduced initial setting times of MTA to 20 min but resulted in decreased CS for MTA containing sodium hypochlorite gel and caused the cement paste to be unworkable with the addition of K-Y jelly [Kogan *et al*, 2006]. Similarly, the addition of 5 % CaCl_2 caused MTA to initially set in 25 min [Kogan *et al*, 2006] but at the expense of decreased CS and set in 35 min according to research conducted by Wiltbank *et al*, 2007. However, with the addition of 3 % CaCl_2 there was no accelerated setting observed [Kogan *et al*, 2006]. The addition of the setting accelerants added to MTA by Kogan *et al*, (2006) caused the CS to decrease significantly compared with MTA without additives. In another study, however, 2 % CaCl_2 was observed to accelerate the setting time of PC to 83 min but also decreased CS [Ber *et al*, 2007]. Larger additions of CaCl_2 (10%) to white MTA and PC reduced the initial setting times significantly to 6 and 19 min respectively [Bortoluzzi *et al*, 2009]. On the contrary, a model cement for MTA, that consisted of 80 wt% PC and 20 wt% bismuth oxide initially set in 2, 1.5 and 1 h with additions of 2, 5 and 10 % CaCl_2 respectively

INTRODUCTION

[Murphy *et al*, 2008] without altering the CS noticeably. With the addition of 20 wt% Plaster of Paris (PoP), the setting times of an MTA-like model system containing 5 and 10% CaCl_2 decreased to just 15 and 12 min respectively [Murphy *et al*, 2008] but decreased the CS significantly and increased RP with time. PoP additions alone and calcium sulphates (CaSO_4) accelerated the initial setting times of MTA-like model systems. Additions of up to 20 wt% PoP or CaSO_4 decreased the setting times of MTA-like model systems below 35 min [O'Beirne *et al*, 2008] but 20 wt% CaSO_4 additions decreased CS and increased RP of MTA-like model root filling materials considerably, unlike other amounts of PoP and CaSO_4 additions which improved CS and RP [O'Beirne *et al*, 2008].

When 1 % CaCl_2 was admixed with 1 % methylcellulose (MC) to form 1 % MC/ CaCl_2 and added to PC, the setting time reduced to 60 min, with no significant change to the CS of the PC control reported [Ber *et al*, 2007]. Higher concentrations of the admixture, MC/ CaCl_2 , did not accelerate the setting of PC as much as 1 % MC/ CaCl_2 but also had little impact on the CS of PC [Ber *et al*, 2007].

Additions of calcium nitrite/nitrate (CN/N) reduced the setting time of PC and grey MTA even further than other accelerants to 7.3 and 7.0 min respectively, however, when added to white MTA, had no significant effect in comparison, setting in 66.7 min [Wiltbank *et al*, 2007]. Calcium formate also had a powerful effect on the setting of PC and MTA, accelerating the setting of PC, grey MTA and white MTA to 9.0 min, 8.7 min and 6.0 min respectively and had little effect on the other physical properties of MTA which were investigated [Wiltbank *et al*, 2007].

The setting time of white MTA was significantly reduced from 3 h to 26 min when a 15 % disodium hydrogen orthophosphate, Na_2HPO_4 , solution was mixed with cements in the liquid

INTRODUCTION

phase and the diametral tensile strength increased when compared with MTA alone [Huang *et al*, 2008].

An accelerated PC was proposed by Camilleri *et al*, (2005b) which was manufactured to exclude gypsum from the cement causing the accelerated setting through 'flash' setting. Cements based on PC that set in 7 min were also investigated as potential restorative materials by Camilleri *et al*, (2006a) and in accordance with improved setting time, these cements had increased mechanical stability indicated by compressive strength testing [Camilleri *et al*, 2007]. Calcium sulpho-aluminate and calcium fluoro-aluminate were incorporated into PC to reduce the setting time to 6 min but like some other accelerants added to PC, reduced CS [Camilleri, 2008]. A similar study concluded that by altering the quantities of the components that comprise MTA (calcium silicate aluminates with or without Mg, Zn or Fe), the setting time can be reduced from 151 min for white MTA to 11 min [Kao *et al*, 2009]. Novel dicalcium silicate-based endodontic materials investigated had similarly low setting times, between 42 and 12 min which decreased with increased CaO content. An endodontic filling material with equal amounts of CaO and SiO had significantly higher CS [Chen *et al*, 2009].

A new endodontic filling material that consisted of calcium compounds, like PC and also contained CaCl₂ was investigated and was found to have a setting time of 50 min which was similar to the setting time of white MTA (70 min) [Asgary *et al*, 2008].

1.7 *Biological properties of MTA and the effect of additives*

1.7.1 *In vivo studies*

MTA was observed to have a dentinogenic effect on pulpal cells of dogs [Faraco *et al*, 2001, Holland *et al*, 2001c, Tziafas *et al*, 2002]. MTA caused functional and cytological

INTRODUCTION

differences in pulpal cells, which resulted in reparative dentine at the surface of mechanically exposed (caused by the production of a class V cavity) dental pulp [Tziafas *et al*, 2002]. Hard tissue bridges were formed on exposed dental pulps treated with MTA [Faraco *et al*, 2001] and hard tissue deposition [Holland *et al*, 2001c] was reported that indicated that MTA is a biologically active substrate for pulpal cells [Tziafas *et al*, 2002]. Regeneration of periradicular periodontium was observed when MTA was used as a root filling material in the teeth of dogs [Regan *et al*, 2002]. Significantly more cementum formed in dogs as hard-tissue healing after periradicular surgery was observed when fresh MTA was compared with set MTA [Apaydin *et al*, 2004].

MTA and PC, implanted into bilateral bone cavities produced in the mandibles of guinea pigs promoted bone healing with minimal inflammatory responses similarly and had been shown to reduce periradicular inflammation [Torabinejad *et al*, 1995e]. In rats, MTA caused acute inflammation around the implant site after 2 weeks and macrophages were present in the radicular pulp [Salako *et al*, 2003]. In contrast, MTA generated only a moderate inflammatory response in subcutaneous connective tissues after 7 days in rats, by evaluating Von Kossa stained sections and a scoring system to determine the level of inflammation [Yaltirik *et al*, 2004]. After 4 weeks of an *in vivo* study in rats, MTA caused the formation of dentine bridges formation surrounding pulps that were capped with the material [Andelin *et al*, 2003] and did not alter the pulp histology *in vivo* in rat molars containing MTA [Salako *et al*, 2003]. A fibrous layer was detected surrounding MTA after 8 weeks of an *in vivo* study in rats with no evidence of inflammation, collagen or mineralised tissue deposition [Kao *et al*, 2006].

MTA was used as a root filling and encouraged the complete regeneration of periradicular periodontium in non-carious human teeth [Torabinejad *et al*, 1999]. Cementum formation on

INTRODUCTION

MTA was a frequent finding during *in vivo* studies [Torabinejad *et al*, 1999], through the induction of cementoblasts to deposit cementum on MTA and PC [Zarrabian *et al*, 2004].

1.7.2 *In vitro* studies

MTA has been reported by many to have similar effects on tissues in *in vitro* studies as that of calcium hydroxide and that the mechanism of action is similar [Holland *et al*, 2001b, Faraco *et al*, 2001]. The release of calcium and phosphorus ions present in MTA [Torabinejad *et al*, 1995b], has been associated with its superior biocompatibility, when compared with other root filling materials [Islam *et al*, 2006a] as these are the principal components of dental hard tissues [Torabinejad *et al*, 1995b]. Also no toxins were found to leak from grey and white MTA during biocompatibility testing of unwashed cements [Camilleri *et al*, 2005].

In simulated biological fluids, MTA released calcium ions (detected with energy dispersive X-ray analysis) which may have contributed to the formation of hydroxyapatite on teeth [Sarkar *et al*, 2005]. An apatite surface was formed on MTA when placed against dentine in the presence of simulated body fluids, Dulbecco's phosphate buffered saline and Hank's balanced salt solution, causing chemical bonding of MTA with dentine [Sarkar *et al*, 2005] known as dentine bridges [Torabinejad *et al*, 1993, Taddei *et al*, 2009a].

1.7.2.1 *Cytotoxicity and proliferative effects of mineral trioxide aggregate*

The cytotoxicity of MTA has been investigated in many *in vitro* studies but yielded conflicting results when different cell types were used. MTA and PC have been shown to generate similar cell reactions as calcium hydroxide [Danesh *et al*, 2006a] for instance, by acting to stimulate cell proliferation [Main *et al*, 2004]. MTA has been shown to be less cytotoxic when

INTRODUCTION

compared with other root filling materials, such as amalgam when in direct contact with cells [Islam *et al*, 2006a]. MTA was found to be non-toxic when the soluble components from 24 h-set MTA were cultured with human periodontal ligament (PDL) fibroblasts and MTA was found to be suitable for use in the apical environment compared with other root filling materials like amalgam and SuperEBA [Keiser *et al*, 2000].

A human osteosarcoma cell line, MG36, was exposed to 1 day old MTA that was sterilised using ultraviolet light. In this study, MTA showed biocompatibility and stimulated bone formation by these cells [Chen *et al*, 2009]. In a Chinese hamster ovary model (L5178Y mouse lymphoma cells), MTA did not cause any cellular death and was not found to be genotoxic [Ribeiro *et al*, 2006]. High survival rates (>100%) were observed for 1 and 7 day old human osteogenic sarcoma (U₂OS) cell cultures exposed to MTA that had been washed in McCoy's culture medium for one day or one week [Kao *et al*, 2006].

Unwashed 24 h-set MTA was reported to have no cytotoxic effects on primary rat dental pulp cells *in vitro* when indirect contact was made but stimulated mineralisation and the increased protein production and bone morphogenic factor (BMP-2) expression compared with dental pulp cells that were not exposed to MTA [Yasuda *et al*, 2008]. Human dental pulp cells cultured from extracted teeth were found to proliferate when exposed to MTA (using indirect contact with cells) that has been pre-immersed in culture medium for 3 days, through the continuous release of calcium ions [Takita *et al*, 2006].

On the contrary, MTA had a slight anti-proliferative effect on three different fibroblast cell lines, L929, BHK2 and RPC-C2A, when 2 day-set unwashed MTA had indirect contact with these cells (like the previous study by Takita *et al*, 2006). MTA affected proliferation to a lesser extent compared with zinc oxide-eugenol and glass ionomer root filling cements [Koulaouzidou *et al*, 2005].

INTRODUCTION

Torabinejad *et al.* (1995c) investigated the cytotoxicity of fresh and unwashed set MTA when placed into cultures with L929 fibroblast mouse-derived cells. It was found that fresh and set MTA gave similar cytotoxicities and were less cytotoxic to cells compared with amalgam, SuperEBA and IRM when the average zones of lysis were quantified [Torabinejad *et al.*, 1995c]. Unwashed white and grey MTA, set for 48hours, were placed into L929 fibroblast mouse-derived cell cultures and cultured for 48hours. This study found MTA to be slightly cytotoxic to fibroblast mouse-derived cells (according to a scoring system, slightly cytotoxic meant that zones of cell lysis were present underneath the sample only) and concluded that grey MTA was less cytotoxic than white MTA [Miranda *et al.*, 2009]. It was also found that there were zones of cell lysis, dead or loosely attached cells surrounding MTA and only further away from MTA were viable fibroblast mouse-derived cells found [Saidon *et al.*, 2003, Miranda *et al.*, 2009]. The same was observed for cultures containing PC [Saidon *et al.*, 2003].

Grey ProRoot MTA was shown to have *in vitro* compatibility with MG-36 and SaOs-2 osteoblast-like cell lines since the toxicity of the material was identified to be less when compared with Super EBA and amalgam [Pelliccioni *et al.*, 2004]. Tooth-coloured ProRoot MTA was found to induce the increased production of DNA in a mouse odontoblast-like cell line and undifferentiated pulp cells and did not induce cell apoptosis or interrupt the cell cycle of these cell types demonstrated with propidium iodide staining followed by flow cytometry [Moghaddame-Jafari *et al.*, 2005]. Immortalised cementoblasts were exposed to the soluble components of MTA (obtained from storage of MTA in Dulbecco's modified Eagle medium) and it was found that high concentrations of soluble components of MTA were toxic to these cells but lower concentrations of the soluble components of MTA had no negative effects on cell morphology, survival and encouraged biomineralisation [Hakki *et al.*, 2009]. Cell proliferation of immortalized murine cementoblasts (OCCM.30) significantly increased after 3 days when placed

INTRODUCTION

in direct contact with white MTA compared with the control [Oviir *et al*, 2006]. Immortalized murine cementoblasts and immortalized keratinocytes (OKF6/TERT1) showed significantly higher proliferation when grown on grey MTA set for 12 days compared with 24 h [Oviir *et al*, 2006].

The cytotoxicity of PC, MTA Angelus and ProRoot MTA were investigated using a human-derived endothelial cells (ECV 304) and it was found that the cytotoxicity of these materials was similar and elevated initially, but with time (72 h) cultures were able to re-establish allowing for cell proliferation [De Deus *et al*, 2005].

1.7.2.1 *Cell attachment*

The attachment of human periodontal ligament fibroblasts on set MTA was observed using SEM and showed that fibroblasts were rounded but closely attached to MTA [Balto *et al*, 2004]. Osteoblasts were seen attached and well-spread on the surfaces of MTA and composite resin called Restorative 2100. However, by comparison, amalgam did not reveal any cell attachment [Zhu *et al*, 2000]. Koh *et al.*, 1998 reported that MTA provides a biologically active substrate for bone-like cells when studies were conducted using osteoblast-like MG36 cells. MTA supported the adherence and growth of immortalized murine cementoblasts [Thomson *et al*, 2003] and these cementoblasts and immortalized keratinocytes grew significantly better on the surface of white MTA than the surface of grey MTA [Oviir *et al*, 2006].

1.7.2.2 *Protein and gene expression*

Unwashed 24 h-set MTA caused a significantly decreased production of proteins by fibroblasts but had only a slight effect on fibroblast proliferation unlike amalgam [Pistorius *et al*,

INTRODUCTION

2003]. Koh *et al* reported that MTA, washed for 72 h in culture medium stimulated the production of interleukins, inflammatory cytokines [Koh *et al*, 1998]. Unwashed MTA that had been allowed to set 7 days prior to culturing stimulated the production of cytokines from osteoblast-like MG36 cells [Koh *et al*, 1997]. MTA caused MC3T3-E1 osteoblasts to proliferate faster and produce a more mineralized matrix gene expression profile, determined using RT-PCR analysis [Tani-Ishii *et al*, 2007]. The expressions of extracellular regulated kinases were stimulated in U₂OS human osteosarcoma cells by MTA, which are associated with apoptosis, cell proliferation and differentiation [Huang *et al*, 2003].

ProRoot MTA and MTA Angelus were found to be capable of stimulating the production of both BMP-2 and transforming growth factor (TGF) β -1 in human gingival fibroblasts when in indirect contact with 72 h-set unwashed MTA with cells [Guven *et al*, 2007]. When periodontal ligament fibroblasts were induced to express alkaline phosphatase, osteonectin and osteopontin when in contact with 24 h-set unwashed and washed MTA, meaning that these cells may have adopted a more osteogenic phenotype [Bonson *et al*, 2004], which are associated with cementum formation [Damaschke *et al*, 2005], concluding that MTA may encourage the formation of cementum [Main *et al*, 2004].

The production of mineralised matrix gene and protein expression by immortalised murine cementoblasts was encouraged by MTA once MTA had been washed three times in 70% ethanol, ultraviolet (UV) light exposed for 20 min and then placed in serum-free culture medium for an additional 72 h. MTA was considered as to be cementoconductive when in contact with this cell type during the study [Thomson *et al*, 2003].

INTRODUCTION

1.7.3 *In vitro* and *in vivo* studies involving modified MTA and PC

The effect of various additives to MTA on its cytotoxicity *in vitro* was investigated by Jafarnia *et al.* (2009) which concluded that the addition of 2 % lidocaine with 1:100,000 epinephrine, saline, 5 % calcium chloride, KY jelly and 3 % sodium hypochlorite gel had no influence on the cytotoxicity of unwashed 24 h-set grey and white MTA, when cultured with L929 fibroblasts for up to 3 days. However, fresh grey and white MTA paste containing 3 % sodium hypochlorite gel increased the cytotoxicity of the material considerably compared with the cytotoxicity of MTA when containing the other additives [Jafarnia *et al.*, 2009]. The cytotoxicity of periodontal ligament fibroblasts was not affected when KY jelly was mixed with MTA compared with the cytotoxicity of cultures containing MTA only [Hernandez *et al.*, 2005].

White MTA mixed with 15 % Na_2HPO_4 that was pre-wetted in 10% foetal calf serum-containing culture medium for 24 h after 24 h of hydration, did not affect the survival rates of a mouse fibroblast cell line (L929) cultured for 1 and 7 days when compared with white MTA only (90% survival) indicating biocompatibility. Cell attachment to white MTA with or without 15 % Na_2HPO_4 was also observed and cells appeared to be well-attached to and spread over the surface of white MTA [Ding *et al.*, 2008]. MTA that was mixed with chlorhexidine was considered to show a tolerate response by tissues during an *in vivo* study conducted by Sumer *et al.* (2006). The addition of chlorhexidine caused a weak inflammatory response *in vivo* during the 60 day study, which was less severe than with pure MTA (without chlorhexidine) and caused the formation of a clear fibrous capsule of connective tissue similar to pure MTA, amalgam and IRM [Sumer *et al.*, 2006].

An accelerated PC that was manufactured without gypsum was reported to change the biocompatibility of PC when in contact with a human osteosarcoma cell line, but cell

INTRODUCTION

proliferation was increased when cells had no direct contact with MTA and accelerated PC, but direct contact resulted in reduced cell growth [Camilleri *et al*, 2005b].

PC containing accelerants (such as calcium chloride) were found to be non-toxic to cells and have the potential to promote bone healing [Kogan *et al*, 2006]. Accelerated PC containing 10 or 15 % CaCl_2 was reported to be non-toxic and SaOS-2 osteosarcoma cells adhered as well to the cement as to MTA [Abdullah *et al*, 2003]. MTA, sterilised using UV irradiation, had higher rates of proliferation of human osteosarcoma cells (MG-63) than the control, proving biocompatibility through the high expression of the bone marker, extracellular signal-regulated kinase [Chen *et al*, 2009]. When exposed to human dental pulp cells, MTA illustrated biocompatibility [Laurent *et al*, 2008, Chen *et al*, 2010] and the focal adhesion kinase in the dental pulp cells was found to be well-distributed [Chen *et al*, 2010] indicating the suitability of the substrate.

1.8 *Sealing properties of mineral trioxide aggregate, modified with additives*

1.8.1 **Dye/ink leakage**

The use of dyes for dental studies was reported by Grossman in 1939, when teeth were immersed into various dyes [Veríssimo *et al*, 2006]. Dye leakage has been used for assessing the sealing ability of root filling materials due to the ease of the method [Tamse *et al*, 1998]. The method had been reported to be unreliable since the longitudinal sectioning of the tooth containing the root canal filling had been a random choice by some researchers [Camps *et al*, 2003]. A variety of dyes and inks have been used to determine the sealing ability of endodontic materials such as MTA. The dyes and inks that have been used for leakage studies include

INTRODUCTION

Rhodamine B, Methylene blue, India ink, Pelikan ink, Silver nitrate and Fuchsin [Parirokh *et al*, 2010]. Many factors were considered to influence dye leakage including the type [Tanomaru *et al*, 2005, Wu *et al*, 1998] and concentration of the dye or ink, the pH of the dye [Roy *et al*, 2001], the treatment of the tooth before placement in dye [Pichardo *et al*, 2006], the length of time MTA was set before placement in dye [Tobón-Arroyave *et al*, 2007], the length of time MTA was stored in dye and the thickness or length of the root canal filling [Valois *et al*, 2004, Coneglian *et al*, 2007, Ölmez *et al*, 2008]. The method used to evaluate the dye leakage into root filling cements was identified as a likely variable for the inconsistency of results obtained for dye leakage studies [Tamse *et al*, 1998] since no standard method for assessment has been established to date.

Methylene blue was used in several dye leakage studies due to the molecular weight being lower than the molecular weight of endotoxin, presenting similar leakage to butyric acid (a product of fermentation produced by anaerobic bacteria) [Kersten *et al*, 1989] but also due to the ease of use and high degree of staining [Veríssimo *et al*, 2006]. Rhodamine B has a similar molecular weight as Methylene blue and was observed that the dye leakage into MTA was significantly less than that of the leakage of Rhodamine B into amalgam and Super EBA [Torabinejad *et al*, 1993]. When the two dyes were evaluated to determine the sealing ability of MTA, less dye leakage was evident into the cement when Methylene blue was used rather than Rhodamine B [Tanomaru Filho *et al*, 2005]. India ink had been proposed as superior for use in endodontic leakage studies since the size of India ink particles range from 0.1-2µm, which would represent the size of some pathogenic bacteria commonly present in root canal infections [Mente *et al*, 2009] and any leakage of the dye within the root filling could also allow bacterial leakage into the endodontic material [Veríssimo *et al*, 2006]. No significant differences were apparent when the sealing ability of gutta-percha and Roth 801 sealer was assessed with dye leakage when

INTRODUCTION

eosin, Methylene blue, black India ink and Procion blue were used and compared with the same leakage method [Tamse *et al*, 1998].

In one study, the addition of calcium chloride improved the sealing ability of the three cements, ProRoot MTA, MTA Angelus and PC when 0.2 % Rhodamine B was used and cements stored for in the dye for 72 h [Bortoluzzi *et al.*, 2006b]. The exposure of white and grey MTA containing 0.12 % chlorhexidine gluconate to India ink for 72 h revealed no significant differences compared with the sealing ability of white and grey MTA without additives [Shahi *et al*, 2007].

1.8.2 Bacterial leakage

Leakage and infiltration studies involving the use of bacteria are believed to be more clinically and biologically representative than dye penetration studies [Timpawat *et al*, 2001]. However, the reliability of bacterial leakage studies is questionable since the experimental set up is delicate and contamination of the broth in the apical chamber could occur if the bacterial broth is spilt and enters the chamber in this way rather than through the tooth, compromising the filling material. The method also is reliant upon the change in turbidity in the broth (used to indicate a poor seal) which could giving false results if bacteria enter the broth through contamination of the experimental methods, mentioned previously [Timpawat *et al*, 2001].

Comparison of the results obtained by bacterial leakage studies proved to be difficult since many strains of bacteria have been used and some of the literature using the same bacteria for the leakage model presents contradictory results [Schäfer *et al*, 2002, Veríssimo *et al.*, 2006]. In a microleakage study, MTA was one of the most effective materials for preventing the microleakage of bacteria compared with other root filling materials such as IRM, amalgam [Main

INTRODUCTION

et al, 2004, Torabinejad *et al*, 1993] and SuperEBA [Maltezos *et al*, 2006] since MTA formed a denser, less porous structure [Pitt Ford *et al*, 1996].

Aerobic bacterial leakage of *Staphylococcus epidermidis* was demonstrated in a 3 mm root canal preparation of white and grey MTA after 30 days using a bacterial leakage model [Montellano *et al.*, 2006] but white and grey MTA prevented leakage of this bacteria after 90 days in another bacterial leakage study [Torabinejad *et al.*, 1995d]. In contrast, MTA prevented the leakage of *Fusobacterium nucleatum* unlike amalgam when an anaerobic bacterial leakage model was used [Nakata *et al.*, 1998]. MTA did not prevent the leakage of salivary microbes in a bacterial leakage study but the study concluded that grey and white MTA acted similarly with regard to preventing leakage [Tselnik *et al*, 2004].

1.8.3 Fluid filtration

The fluid filtration system evaluates pressure-driven fluid transport in root fillings. The air bubble is displaced when fluid enters the root canal filling and the distance in which the bubble had displaced was measured to compare the sealing ability of materials [Javidi *et al*, 2008]. The microleakage of MTA, amalgam, IRM and Super-EBA were evaluated using the fluid filtration method and suggested that amalgam showed the most microleakage compared with the other root filling materials MTA, IRM and Super-EBA, which had similar microleakage recorded [Fogel *et al*, 2001]. 10% CaCl₂ addition to MTA and PC showed to reduce microleakage when analysed by flow porometry in comparison to MTA and PC without additions of CaCl₂ [Hong *et al*, 2008]

1.8.4 Glucose and protein leakage

Glucose leakage into root filling materials was introduced based on the filtration method (see section 1.12.3) and used due to the low molecular weight of glucose, being a nutrient for the

INTRODUCTION

survival of bacteria. Therefore, should no leakage be attributed when using glucose as a tracer, the material would be considered to possess excellent sealing ability in theory [Xu *et al*, 2005]. The sealing ability was investigated using a glucose leakage model to establish the sealing ability of MTA for the repair of furcation perforations with and without an internal matrix of calcium sulphate. Calcium sulphate statistically decreased the sealing ability of MTA in this study; however, conflicting results had been obtained by other researchers [Zou *et al*, 2008]. A protein leakage model had also been used to investigate the sealing ability of MTA and in this study it was concluded that the thickness of the root canal material is an important factor for the sealing ability of the material [Valois *et al*, 2004]. MTA exposed to more acidic environments had more protein leakage compared with environments that were more alkaline [Saghira *et al*, 2008].

1.9 *Antimicrobial properties of modified mineral trioxide aggregate*

The antimicrobial and antifungal action of MTA has been investigated using agar diffusion plates and showed that MTA inhibited the growth of the following bacteria: *Enterococcus faecalis*, *Staphylococcus epidermis* [Al-Nazham *et al*, 2003, Leimburg *et al*, 2004], *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Candida albicans* [Al-Nazham *et al*, 2003, Al-Hezaimi *et al*, 2006] and *Enterobacter aerogenes* [Leimburg *et al*, 2004] in immature apices. However, MTA was found to be ineffective at inhibiting the growth of *Escherichia coli* [Al-Nazham *et al*, 2003]. Grey MTA resisted the microleakage of *Actinomyces viscosus* for 70 days [Al-Kahtani *et al*, 2005].

The antibacterial and antifungal effects of MTA are thought to be a result of the high pH, which is caused by the generation of calcium hydroxide as a hydration product of MTA during the setting reaction [Parirokh *et al*, 2010]. The pH of MTA was reduced by the addition of

INTRODUCTION

calcium chloride [Bortoluzzi *et al*, 2006a], thus potentially affecting the anti-microbial action of MTA [Kogan *et al*, 2006]. Other additives to PC that have been studied include chlorhexidine gel and NaOCl gel and K-Y jelly which have been found to improve the antimicrobial action of cement whilst maintaining the alkalinity of PC and MTA [Kogan *et al*, 2006]. The antimicrobial effect of white MTA was also enhanced by the incorporation of chlorhexidine gluconate (0.12 %) [Kogan *et al*, 2006, Bortoluzzi *et al*, 2006a].

1.10 *Other properties of mineral trioxide aggregate*

The pH of MTA has been shown to change from 10.2 to 12 during setting and remained constant 3 h afterwards [Torabinejad *et al*, 1995b] and it is suggested that the high alkalinity of MTA promoted its anti-microbial action [Kogan *et al*, 2006]. An acidic pH may adversely affect the hydration behaviour since the pore solution will not become alkaline causing the formation of different hydration products and the physical properties of hydraulic cements such as MTA and PC would change as a consequence [Kogan *et al*, 2006].

Some researchers have reported that MTA possesses poor handling characteristics due to the fine cement powder being difficult to mix with water to form a homogeneous paste [Ber *et al*, 2007]. However, others have indicated that MTA is easy to mix to a homogeneous paste [Chng *et al*, 2005, Islam *et al*, 2006, Danesh *et al*, 2006a, Torabinejad *et al*, 1993]. MTA has been reported to become more viscous in the presence of excess liquid, which could occur during insertion into the root and affect the setting properties [Chng *et al*, 2005, Islam *et al*, 2006] and potentially the longevity of the cement.

Endodontic materials need to be more radiopaque than the surrounding tissues to enable easy observation using X-rays [Torabinejad *et al*, 1995b]. The minimum required radiopacity for

INTRODUCTION

a root filling material [ISO 6876:2001] is equivalent to 3 mm of aluminium [Danesh *et al*, 2006a]. The mean radiopacity of MTA is equivalent to 7.17 mm of aluminium [Islam *et al*, 2006a], which is more radiopaque than PC, due to MTA containing ~20 wt% Bi_2O_3 as a radiopacifying agent [Danesh *et al*, 2006a].

1.10.1 Solubility and stability of mineral trioxide aggregate

Solubility and sealing ability are linked as soluble materials may not provide a long term seal due to material being removed through dissolution, therefore not acting as a long-term barrier in the area where it has been placed. The sealing ability of a material will thus be compromised should the material be very soluble [Danesh *et al*, 2006]. Set MTA has a low solubility when in contact with tissue fluids, as shown in *in vitro* studies, where it was found that white and grey PC were more soluble than the PC that comprises grey MTA [Islam *et al*, 2006a, Danesh *et al*, 2006, Torabinejad *et al*, 1995b]. Differences in the phases formed through the hydration and setting of PC, due to the cements different chemical compositions [Taylor, 1997], may explain differences in the solubility of PC [Islam *et al*, 2006a]. The degree of solubility and porosity increased significantly when the water to powder ratio of grey MTA was increased [Islam *et al*, 2006a], due to inclusion of microscopic air bubbles and excess water (not consumed in the setting reaction) mixed into the paste, both contributing to porosity [Fridland *et al*, 2003].

1.11 *Additives: Calcium sulphates*

There are three basic calcium sulphates which are anhydrous calcium sulphate (CaSO_4), calcium sulphate hemihydrate; Plaster of Paris ($\text{CaSO}_4 \cdot \frac{1}{2}\text{H}_2\text{O}$) and calcium sulphate dihydrate; gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$). Calcium sulphates have been used in the treatment of dental diseases such as periodontal disease, alveolar bone loss and endodontic lesions as calcium sulphates provide a direct source of calcium ions that promote the healing and regeneration of bone [Orsini *et al*, 2004]. However, calcium sulphates set to form a brittle material which contains many pores and has a low CS [Orsini *et al*, 2004] and the final product of hydration, gypsum, is very soluble. The significance of this is that if added to MTA, there could potentially be an increase in the solubility of MTA and also there could be a potential decrease in the compressive strength of MTA.

1.12 *Aims of the project*

Since MTA is an expensive material and the composition of the commercial material cannot be controlled, it was decided that a PC based material (with similar properties to MTA) was to be developed as model cement representing MTA for the bulk of the research carried out. A model system was also be designed for control of the cement composition. The aim of the investigation was to accelerate the setting of the PC-based model system by modification with additives with the intention of addressing the limitation of MTA, being its overly-long setting time. The intention was to incorporate additives that would reduce the initial setting time of MTA and a model system for MTA as much as possible, to more idealistic times without altering the properties of the material which have caused the success of the material for use in endodontics (biological and sealing properties). Additives that accelerated the setting of the model system

INTRODUCTION

were also added to MTA to confirm the validity of the model system and enable comparison with commercial MTA.

The focus of the project was to accelerate the setting of a dental PC-based root filling material by incorporating setting modifiers whilst maintaining or improving the mechanical strength of the cement and the amount of porosity in the cement structure. Successful setting accelerants could then be applied to commercial MTA to decrease its long setting time whilst maintaining mechanical properties. The additives proposed would be subjected to long term storage in solutions in order to investigate the effect of *in vitro* ageing on the mechanical properties of the material. This may give an insight into the longevity of the modified material as a root filling cement and the microstructural changes to the cement with long term storage which has the potential to negatively affect the sealing capabilities of MTA.

The additives proposed would be subjected to *in vitro* testing to investigate the biocompatibility of MTA with additives i.e. investigate the level of toxicity of modified cements on cells. Since there are conflicting reports published in the literature with regard to the *in vitro* effect of MTA, the aim of the studies are to provide clarity to what is actually happening to cells when in contact with MTA, which will provide a basis upon which the effects of modified MTA may be compared. This may require exploring all the possible variables to ensure reliable results and conclusions are being drawn, which will include varying the experimental set ups and the use of different cell types.

The sealing ability of MTA is vital for endodontic applications and therefore the effect of additives on the sealing ability of MTA and PC were investigated. Since no standardised method for dye leakage had been established previously, the sealing ability of MTA containing additives was investigated by highlighting the variables of the dye leakage method. The dye or ink used, the concentration, the length of time the root filling had been allowed to set before exposure to

INTRODUCTION

dye/ink and the length of time of dye or ink exposure to ensure comparison of dye leakage were investigated. The results from the dye leakage studies would then be used to investigate the sealing ability of modified commercial MTA in a tooth model. The tooth type used for the study, preparation of the cavity that will be filled with root filling material, i.e. the length and type of storage, root canal preparation and the length of the root canal filling were considered as these variables that needed to be standardised to develop a reproducible and reliable method for determining the sealing capacity of modified MTA.

2 MATERIALS AND METHODS

A model for mineral trioxide aggregate (MTA), used as an endodontic filling material, needed to be established in order to control composition and also because MTA is an expensive material and relatively large quantities of the material needed to be used during the study.

2.1 Cements and powders used

Three different Portland cements (PC) (Blue Circle, LaFarge, UK) were investigated as the main components for the model system: two grey PCs, Mastercrete (MC) and Original Portland cement (OPC) and one white PC, Snowcrete (SC). A model was generated for grey MTA which consisted of 80 wt% MC mixed with 20 wt% bismuth oxide, Bi_2O_3 (Sigma Aldrich, UK or Acros Organics, UK), used to enhance the radiopacity of MTA. A model for white MTA was produced consisting of 80 wt% SC and 20 wt% Bi_2O_3 .

Three calcium sulphates were investigated as potential setting accelerants for MTA. Anhydrous calcium sulphate (CaSO_4), calcium sulphate hemihydrate or gypsum (Gyp) (Sigma Aldrich, UK or Acros Organics, UK) and calcium sulphate hemihydrate, commonly referred to as Plaster of Paris, PoP, (Crystacal R Plaster, BPB Formula, UK) were added to the model system for grey MTA, a potential model for white MTA (containing SC) and grey MTA (Dentsply ProRoot MTA Original, UK) in 5, 10, 15, 20 and 30 wt%. PC content varied according to the amount of calcium sulphate being added, whilst Bi_2O_3 content was kept constant (20 wt%).

MATERIALS AND METHODS

2.1.1 Powder and cement paste preparation

Cement powders and additives were sieved (Endecotts Ltd, London, UK) to 500 μm (size of aperture) then ground using a pestle and mortar to minimise the presence of agglomerates within the cement mixtures. Cement powders were hand-mixed using a spatula with distilled water at powder to liquid ratios (PLR) of 2.5, 3.3 and 4.0 g/ml.

2.1.2 Sample generation

Cement pastes of PLR 2.5, 3.3 and 4.0 g/ml were placed into a polytetrafluoroethylene (PTFE) split mould to produce cylindrical samples with aspect ratios of 2 (6 x 12 mm, diameter and height) or 1.5 (4x6 mm, diameter and height). Following mixing, cement samples were stored at 37°C until initially set (6 h) and then extracted from the mould. Cements (10 samples) were then stored in 20 ml deionised double distilled water at 37°C for 10 days to simulate (but to an extreme effect) the effects of moisture that the cement would come into contact with on placement and whilst present within the root.

2.1.3 Sterilisation of powders and set cements

2.1.3.1 *Dry heat treatment of powders*

Powders were prepared as previously described (section 1.1.1) and double wrapped in foil to be heated in an oven at 120°C for 1 h.

2.1.3.2 *Autoclaved cement samples*

Cement samples were produced (as described in section 2.1.2) and extracted from moulds 6 h after setting before being wrapped in foil (cements placed into autoclave without being pre-wetted: dry). Alternatively, cements were wetted with distilled water then double wrapped in foil (wet autoclaved cements). Samples were autoclaved at 120°C at 15 kPa for 30 min.

2.1.3.3 *Gamma radiation*

Powders were prepared as previously described and gamma irradiated with 25 Gy according to the ISO standard (ISO 11137-1:2006).

2.1.1 **Samples for cell culture**

Sterilised cement powders were mixed with water at a PLR of 3.3 g/ml and then placed into sterile single PTFE split moulds (autoclaved) in a laminar flow hood (to maintain sterility) to produce cements with the following dimensions: 6 mm diameter and 3 mm height. Cells were also exposed to freshly mixed cement paste (0.2 g) which was either placed into the centre of 6-well culture plates or on thick circular glass slides that had a diameter of 13 mm inside the 6-well culture plates for scanning electron microscopy (SEM) analysis of samples and left to set for 30 min. 2 g cement paste was placed into 24-well culture plates to cover the entire base of the well and a flat surface was created by gently vibrating the wells using a vibrating unit (Miximactic, Jencons Scientific Limited) and samples were left to set for 24 h in a humidified incubator (IG150, Jouan, France) in an atmosphere of 5 % CO₂ at 37°C.

2.2 *Characterising cements*

2.2.1 **Setting time**

The initial setting times of cements were obtained using a standard Gillmore needles test [ISO9917-1:2003] at 23°C. The cement pastes (n=3) were placed into 24-well cell culture plates to provide an adequate surface area (and thickness of the cement paste) for needle indentation into the cement. Needles were indented into cement pastes at 5 min intervals for the small Gillmore needle (diameter 0.21 mm, mass 113.4 g) and every 30 min when the larger needle was used (diameter 0.11 mm, mass 453.6g). The principles of the Gillmore needles test relates to the mass of the needle and surface area of the head of the needle, which corresponds to the force that each needle applies to a surface. The point at which a cement paste can no longer be indented by the force provided by the small Gillmore needle has been characterised as the point at which the cement paste is initially set and recorded (when measuring the length of time taken to reach this point) as the initial setting time of cement. The larger needle was used to measure the final setting time of the cement. The lid of the 24-well plate covered cements between indentations to prevent dehydration of the cement paste surface during setting.

2.2.2 **Compressive strength**

Compressive strength (CS) was measured as a bulk material property of the material and also used to detect any changes in the material following the incorporation of additives. Cement samples (n>10) were removed from storage after 10 days setting, weighed and the diameter and height of each sample were measured using a digital Vernier (Linear Tools, UK). Compressive loads at failure were obtained using a mechanical testing machine (Instron1185, UK) with a crosshead speed of 1 mm/min. Wet CS were calculated using the equation:

$$CS = \frac{L}{A} = \frac{L}{\pi \cdot \left(\frac{d}{2}\right)^2}$$

CS = Compressive strength (MPa)

A = Cross-sectional area of cylindrical sample

L = Load (N)

d = Diameter (mm)

2.2.3 Weight loss experiment

Large cement fragments, obtained following CS testing, were stored in a desiccator to dry and weighed twice a week for one month to determine the dry mass for weight loss calculations. The large fragments were considered to be dry when the weight of fragments no longer changed. Calculations to determine the apparent wet density were made using the dimensions of samples and wet weight. The following equation was used to determine the dry density of samples.

$$\rho_{dry} = \frac{W_D}{W_W} \times \rho_{wet}$$

ρ_{dry} = Apparent dry density (g/cm³)

W_D = Dry weight of fragments (g)

ρ_{wet} = Apparent wet density (g/cm³)

W_W = Wet weight of fragments (g)

2.2.4 Calculation of relative porosity

Relative porosity (RP) was measured as an indicator for the potential sealing ability of cements [Fridland *et al*, 2003] and to aid the interpretation of CS data. Large dry fragments

MATERIALS AND METHODS

yielded from cement samples following CS testing were used to obtain RP values. Dry cement fragments were placed into a helium pycnometer (Accupyc 1330, Micromeritics, USA), which calculated strut densities by measuring the volume of helium required to fill the chamber containing fragments and the mass of the cement fragments. Combined with dry density calculations, the RP (%) was obtained using the following equation.

$$RP = 1 - \frac{\rho_{dry}}{\rho_{strut}}$$

ρ_{dry} = Apparent dry density (g/cm³)

ρ_{strut} = Strut density (g/cm³)

2.2.5 Scanning electron microscopy

2.2.5.1 *Sample preparation*

Cells from cultures were fixed in either 2.5 % glutaraldehyde buffer containing 0.1 M sodium cacodylate in or 6 % paraformaldehyde in 0.1 M PBS for 30 min or 1 h respectively and stored in the refrigerator at 4°C. Samples were later removed from the fixative solution and dehydrated by being placed into increasing concentrations of ethanol (20, 30, 40, 50, 70, 90, 95 (x 2) and 100%). Samples were later dried from CO₂ with a critical point dryer (E3000 critical point dryer, Bio-Rad Laboratories Ltd, UK).

Critical point dried glass slide cultures and cements with adherent cells, and air-dried cement fragments were fixed to aluminium stubs (Agar Scientific Ltd., Essex, UK) using adhesive carbon conducting tags (Agar Scientific Ltd., Essex, UK) then gold sputter coated (Emitech K550X, Emitech Ltd., Ashford, Kent, UK) for 2 min (25 mA voltage) to provide a conductive surface. Samples were examined using scanning electron microscopy, SEM (Jeol

MATERIALS AND METHODS

840A and 5300LV, UK), using an accelerating voltage of 10kV. Macro- (x 200 to x 500 magnification), micro- (x 2000 magnification) and crystal structures (x 5000 magnification) of cements were examined using images captured with Semaphore computer software (Digital slow scan image recording system, Version 4.01, LEAD Technologies, Jeol, Sundbyberg, Sweden). Cell attachment to cement samples and glass slides, cultured for various time periods, were observed using SEM.

2.2.6 X-ray diffraction analysis

Dry cement samples ($n > 3$) for X-ray diffraction analysis (XRD) were crushed using a vice and ground to a powder using a pestle and mortar. XRD analysis was performed to identify the composition of the crystalline components in the set cement. A Bruker diffractometer was used in transmission mode with a germanium primary beam monochromator position sensitive detector (Siemens D5005 Diffractometer Bruker AXS Karlsruhe, Germany). The identification of crystalline phases was carried out by comparing the diffraction patterns with known diffraction patterns from the Joint Committee on Powder Diffraction Standards database (JCPDS). Patterns were assigned according to the match with known diffraction patterns and phases labelled.

2.2.7 Dissolution kinetics of the additives

The initial dissolution kinetics of the additives PoP, CaSO_4 and Gyp were investigated using a calcium selective electrode [detectION, Nico Scientific, Pennsylvania, USA] to measure the concentration of calcium (Ca^{2+}) ions every 5 min for 1 h. 0.25g of PoP, CaSO_4 or Gyp were

MATERIALS AND METHODS

added to 100 ml distilled water and stirred throughout the experiment. The first measurement was taken 1 min after powders were added to the distilled water.

2.3 *Cell culture mediums and solutions*

2.3.1 **Medium**

Minimum essential medium (alpha modification), α -MEM, was prepared in accordance with the manufacturer's instructions (Sigma Aldrich, UK) and supplemented with 2.2 g sodium bicarbonate (Sigma Aldrich, UK) per 1000 ml of α -MEM. Following pH adjustment to 7.3, the medium was filter sterilised under pressure through a cellulose nitrate membrane with a pore size of 2 μ m (Whatman Schleicher & Schuell International Ltd, Maidenstone, England, UK).

2.3.1.1 *Transport medium*

The medium was supplemented per 20 ml with 500 μ l HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (Sigma Aldrich, UK), 2 ml penicillin/streptomycin (Sigma Aldrich, UK), 200 μ l amphotericin (Sigma Aldrich, UK) and used to store rat femora prior to cell extraction.

2.3.1.2 *Growth medium*

The constituents of the supplemented medium were 20 ml α -MEM, 3 ml foetal calf serum (Sigma Aldrich, UK), 500 μ l HEPES (Sigma Aldrich, UK), 200 μ l penicillin/streptomycin (Sigma Aldrich, UK), 24 μ l amphotericin (Sigma Aldrich, UK) for bone marrow stromal cell (BMSC) cultures and 20 ml α -MEM, 2 ml foetal calf serum (Sigma Aldrich, UK), 500 μ l HEPES (Sigma Aldrich, UK), 200 μ l penicillin/streptomycin (Sigma Aldrich, UK) to feed osteoblastic and fibroblastic cultures.

MATERIALS AND METHODS

2.3.1.1 *Cell dissociation solutions*

0.1 M phosphate buffered saline, PBS (Sigma Aldrich, UK), was used to wash away non-adherent or dead cells and remove any depleted or old culture medium that may have remained in culture flasks or dishes containing adherent cells. PBS was later removed and replaced with 0.25 % trypsin/ 0.2 % EDTA (Ethylenediamino-tetraacetic acid), Sigma Aldrich UK, for the removal of adherent cells for counting (using a haemocytometer, method fully described later in section 2.9.3) or passaging.

2.3.2 **Cell extraction**

2.3.2.1 *Bone marrow stromal cells*

The femora of neonatal or 6 week old (young adult) albino Wistar (250 g) male rats were removed to extract bone marrow stromal cells (BMSC) using an established method [Maniopoulos *et al*, 1988]. A surgical scalpel (Swann Morton, Sheffield, UK) was used to cut through the skin and gain access to the underlying tissues attached to the femora to enable removal of the femora. Femora were stored in transport medium before soft tissue was cleaned from the femora, using a scalpel and forceps whilst in a petri dish containing transport medium. The epiphyses of femora were removed, performed by using sterile wire cutters for the removal of epiphyses of adult femora and the epiphyses of neonatal femora were removed using a scalpel. The femoral marrow was extracted by flushing the marrow cavity with growth medium into sterile universal containers, using a syringe with an 18 (Tyco healthcare, Northern Ireland) or 25gauge needle (Terumo, Belgium). The medium containing BMSCs was centrifuged for 3 min at 1200revs per min (rpm) to obtain a cell pellet and replaced with fresh growth medium. Following centrifugation, the supernatant was removed and the cell pellet was thoroughly mixed

MATERIALS AND METHODS

with growth medium to disperse cells evenly then transferred into culture flasks (25 or 75cm³) for storage in a humidified incubator containing 5 % CO₂ at 37°C (IG150, Jouan, France). Culture medium was changed every two to three days. BMSCs were used for experimental cultures before reaching passage five to maintain the primary BMSCs phenotype [Derubeis *et al.*, 2004].

2.3.2.2 *Primary osteoblasts and fibroblasts*

Neonatal albino Wistar rats (one day old) were sacrificed by cervical dislocation and stored on ice to minimise tissue degradation. The calvaria was exposed by making two incisions into the skin covering the skull, using scissors and forceps (decontaminated with 70% ethanol) and pinning the skin to the polystyrene dissection board that the rat was laid upon, using dissection pins. The calvaria were removed by cutting around the bones comprising the skull (figure 3(a) on the following page). Calvaria were placed into 90 mm bacteriological petri dishes (AppletonWoods, UK) containing enough medium to cover the calvaria in order to keep tissues hydrated and minimise tissue degradation. Calvaria were trimmed using scissors and the parietal bones removed by cutting along the parietal side of the sagittal suture (figure 3(b and c)). The periosteum was removed from the parietal bones using forceps to locate and separate the periosteum from bone in order to obtain fibroblastic (periosteal) whilst osteoblastic cultures were established from finely minced parietal bones. The periosteum and parietal bones were cut into small pieces (figure 3(d)) and cultured in supplemented α -MEM separately in 60 mm dishes (3-4squares of tissue per dish) for 5-7 days in a humidified incubator with 5 % CO₂ at 37°C (IG150, Jouan, France) to allow for cell and tissue adherence to culture plates. Culture medium was changed three times a week until confluence was reached and cultures were used before reaching passage five.

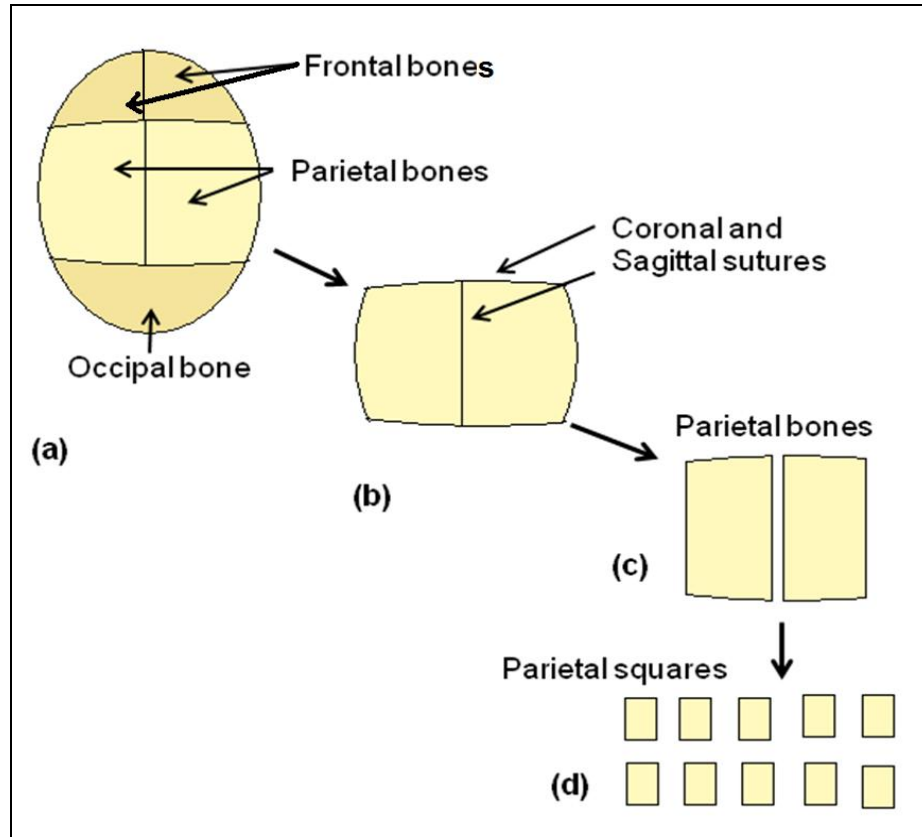


Figure 3: A schematic diagram to represent the preparation of the calvaria and removal of parietal bones to attain osteoblastic and periosteal cultures. The calvaria were trimmed as represented by (a), and then parietal bones separated from occipital and frontal bones (b). The parietal bones were cut along the parietal side of the sagittal suture (c) and after the removal of the periosteum, cut into squares to attain primary osteoblastic cultures (d).

2.4 *Characterisation and generation of a control (model) cement*

2.4.1 **Selection of Portland cement for model system**

MC (Mastercrete Original), OPC (Ordinary Portland cement) and SC (Snowcrete) were investigated for suitability as the main component of the model system. Cements were prepared as described in section 2.1.1-2.1.3, to produce samples with PLR of 2.5 g/ml, 3.3 g/ml and 4.0 g/ml. CS testing and RP calculations for set cements were undertaken using the methods described in sections 2.2.2 and 2.2.3 and were carried out to find the cement which provided the highest CS and lowest RP since cements with these attributes would be likely to enhance the

longevity and sealing properties of the material. Cement powders were examined using SEM to compare particle size and distributions of the cement powders.

2.4.2 Selection of bismuth oxide used as a component of the model system

Two bismuth oxides from different manufacturers were investigated to find the most suitable radiopacifying component for the model system, with regard to maintaining a high CS and a low RP in the PC. Cement powder containing 80 wt% MC and 20 wt% Bi_2O_3 (control) was prepared and a cement paste at a PLR of 3.3 g/ml produced as described in section 2.1.1. Cement samples (10 samples) containing either Bi_2O_3 manufactured by Sigma Aldrich, UK (Bi_2O_3 Sigma) or Acros Organics, UK (Bi_2O_3 Acros) were produced to identify whether the particle size of Bi_2O_3 influenced the CS and RP of the cement.

2.4.3 Effect of time of mould storage on stability and structure of cement

To improve the method of sample production for CS testing a study was conducted to evaluate the effect of extracting samples from the PTFE split mould at various time points, on the CS and RP. Samples extracted after short setting time periods may be advantageous for mass production but could result in weaker samples (decreased CS and increased RP). Alternatively, the effects of samples setting in the mould for long periods of time may cause the dehydration of samples, which could result in decreased CS and increased RP. Decreased CS and increased RP caused by alterations in the methodology of sample production could cause difficulty in determining changes in CS and RP with the addition of setting accelerants and therefore had to be taken into consideration. Thus, control cement samples (80 wt% MC, 20 wt% Bi_2O_3 , PLR 3.3 g/ml) were prepared and produced as described in sections 2.1 and 2.2.2-2.2.4 and extracted after

6 hours, 1 day, 3 days and 7 days of setting and then placed into double distilled water for 10 days at 37°C.

2.5 *Addition of calcium sulphates to the model system*

2.5.1 **Effect of additives on the microstructure of the model system**

Cement formulations (containing additives PoP, CaSO_4 and Gyp) were examined before hydration in powdered form and after hydration (as set cements) using SEM. Set cements containing calcium sulphates (5-30 wt%) were analysed using XRD.

2.5.2 **Selecting calcium sulphates**

The additives, i.e. different CaSO_4 and Gyp, were examined in order to determine whether these calcium sulphates from different manufacturers differed in particle size and could cause changes to the setting time and mechanical properties of the set cement. Different grades of PoP (from different manufacturers and with varying density) were also added to MC to determine the effects on the setting time.

2.6 *Addition of Plaster of Paris to mineral trioxide aggregate*

To reduce the amount of commercial MTA used to generate a large sample size (10 samples) for reliable CS and RP results, samples with an aspect ratio of 1.5 were produced. More samples could be generated with less cement powder consumption when a mould that produced samples with an aspect ratio of 1.5 rather than the use of a mould that produced cement samples

MATERIALS AND METHODS

with an aspect ratio of 2. 10 wt% and 20 wt% PoP additions were incorporated into grey MTA, the model system for grey MTA (consisted of 80 wt% MC, 20 wt% Bi₂O₃) and the potential model for white MTA (80 wt% SC, 20 wt% Bi₂O₃) to investigate the effects on CS and RP. Microstructural changes to the outer and fracture surfaces of MTA and model cements containing PoP were examined by SEM.

2.7 *Long term solubility and stability of the PoP-modified MTA-model*

Control cement samples (MC or SC) and cements containing 30 wt% PoP were produced using gamma irradiated powders (see section 1.1.2.3) and stored in 20 ml distilled water or supplemented α -MEM, which was changed on a weekly basis. The weekly release of calcium ions (using a calcium selective electrode, see 2.3.7) and the pH (pH211 microprocessor, HANNA Instruments, Bedfordshire, UK) were measured. CS of cements was measured after 10 days, 1, 3, 6, 9 and 12 months of storage and RP of large fragments from these cements determined as described in sections 2.3.2 and 2.3.4.

2.8 *In vitro effect of the PoP-modified MTA-model*

2.8.1 Cell morphology

Sterilised cement samples and fresh cement pastes were placed into the centre of circular glass slides (13 mm diameter, 100 μ m thickness or 22 mm diameter, and 160 μ m thickness) in 6-well culture plates. Adult and neonatal BMSC, fibroblasts and osteoblasts were seeded directly onto cements and glass slides or set cements were placed onto established cell cultures containing 1.0×10^5 cells per well. 1.6×10^4 cells were cultured for 1, 2, 3, 4, 6, 8, 10 and 14 days and

MATERIALS AND METHODS

cultures viewed using phase contrast microscopy (Nikon Eclipse TE300, Tokyo, Japan) and images were captured using a Nikon CoolPix 880 camera (Nikon Corporation, Tokyo, Japan). Scale bars on images were produced with the aid of a stage micrometer (Agar Scientific, England, UK). Cells attached to cement in cultures were analysed further at higher magnifications using SEM. Images of culture glass that had been in cultures containing modified and unmodified model cements and MTA were captured with Semaphore computer software to examine inhibition zones of cell growth which were measured using Semaphore computer software also.

2.8.2 Cell counts

Adult and neonatal BMSC, fibroblasts or osteoblasts were seeded into 6-well culture plates, 24-well culture plates and 96-well culture plates (for different studies) at a density of 1.6×10^4 cells per well. Cultures were maintained for 1, 2, 3, 4, 6, 8, 10, 12 and 14 days. The medium from cell culture wells plates was removed after 3 days and the pH measured. Cultures were washed with 0.1 M PBS to remove any non-adherent cells and dilute any medium that may still be present in the cultures. Cement samples from cell cultures were removed and placed into universal containers containing 0.25 % trypsin-0.02 % EDTA solution (Sigma Aldrich, UK) to detach any adherent cells from cement samples. PBS was removed from cell culture wells and replaced with the trypsin-EDTA solution (Sigma Aldrich, UK) to detach cells adhered to culture dishes for counting. The trypsin-EDTA solution was deactivated with supplemented α -MEM once all of the cells had detached from culture plates identified using phase contrast microscopy (Nikon Eclipse TE300). Trypsin-EDTA solution breaks down proteins responsible for cell attachment to substrates, however, if detached cells were in contact with a trypsin-EDTA solution for extended periods, the cells may become damaged and the cell viability may become

MATERIALS AND METHODS

compromised [Butler, 2004] hence the reason for deactivating a trypsin-EDTA solution once cells have detached from the substratum. Solutions containing cells detached from cell culture wells and solutions containing cells detached from cements were centrifuged to obtain cell pellets that were resuspended in either 0.5 or 1 ml of medium. Cell suspensions were diluted further (1:1 ratio) with 0.2 % trypan blue and 100 μ l pipetted onto an improved Neubauer haemocytometer (Agar Scientific, UK) to determine cell numbers (converted to cell densities when analysed in the results sections) and viability.

2.8.3 Live/dead staining

1.6×10^4 cells per well were seeded onto cements in 24-well culture plates. Cultures were analysed using fluorescent dyes to observe the viability of cells according to the distance the cells that adhered from cements after 3 days of culturing. 5 μ l (per 1 ml of culture medium) calcein-AM, calcein acetoxymethyl (Sigma Aldrich, UK), was placed directly into culture medium after 3 days of culturing and incubated at 37°C for 20 min. Propidium iodide (PI) (Sigma Aldrich, UK) was directly inserted into culture medium (20 μ l PI per 1 ml of culture medium) and cultures were further incubated at 37°C for 5 min. Calcein-AM stains living cells (which appeared green using fluorescence microscopy) and PI binds to the nucleic acids in the nuclei of cells that have incomplete cell membranes (red nuclei and rest of cell green) or dead cells (completely red). Images of live/dead cells were captured using a Nikon CoolPix 880 camera (Nikon Corporation, Tokyo, Japan).

2.8.4 Washing of cements to be placed into cell culture

80 wt% MC 20 wt% Bi₂O₃ cements were produced for cell cultures as described in section 2.1. After 1 day of setting, cements were placed into 10 ml supplemented α -MEM, 0.1 M

MATERIALS AND METHODS

PBS, 70% ethanol or double distilled water and stored at 37°C in a humidified incubator or room temperature (cements in 70% ethanol only) for 24 h. Control (cement) samples for the experiment were stored in universals without storage solutions at 37°C in a humidified incubator for 24 h. Cells were seeded directly onto cements in 24-well culture plates or control cultures at a density of 1.6×10^4 cells per well. Cell counts were undertaken after cultures were incubated for 3 days (see section 2.9.2).

2.9 *Sealing ability of PoP-modified MTA and model cement*

2.9.1 **Establishing a method for dye leakage studies**

A method for determining the sealing ability of cements using dye leakage studies needed to be developed as according to the literature various methods have been proposed, mainly using subjective scoring systems. Since several different dyes or inks have previously been used for dye leakage studies (for example Methylene blue, Rhodamine B and India ink) and generated rather variable results, it was necessary to determine which dye or ink and concentration would be used to investigate the sealing ability of the model system and MTA either with or without PoP additions. Some dyes were selected on the basis that the molecular weights of the dyes were similar to the molecular weights of dyes previously used for dye leakage studies (Toluidine blue, Safranin O and Crystal violet). Other aspects of dye leakage studies had to be investigated in order to standardise the method since the length of time that samples were set before being placed into dyes or inks and the length of time that cements were stored in dyes or inks varied according to the literature.

MATERIALS AND METHODS

Investigations were performed on samples with 6 x 12 mm (diameter, height) consisting of 80 wt% MC and 20 wt% Bi_2O_3 and cements containing 10 and 30 wt% PoP, which were produced as described in section 2.1. After initially setting for 1, 2, 3 and 10 days, cements (10 samples) were placed into universal tubes containing 20 ml of dye (0.2, 0.5, 1, 2 or 5 % concentration) or 100% India ink (Sanford Higgins, Lakewood, NJ) and stored for 1, 3 or 7 days at 37°C. The dyes investigated were Methylene blue, Rhodamine B, Safranin O, Toluidine blue and Crystal violet, CV (Sigma Adrich, UK). Excess dye or ink was wiped from the surface of cements that had been extracted using a paper towel. Following 3 days of air-drying, samples were cut longitudinally into halves (measured using a digital Vernier) using an Isomet low speed saw (Buehler, Illinois, USA) with a diamond blade (MetPrep Ltd, Coventry, UK) in order to measure the extent of the dye or ink permeation that was revealed. The cut surfaces of the cement halves were polished (dry) by hand with silicon carbide paper (Struers A/S, Denmark, Europe) 80, 500, 1000 and 2400, to produce flat surfaces. The cut surface of the sample was polished to provide full contact with scanner (Epson Perfection V200 Photo, Epson Ltd, UK) using a maximum resolution of 2400 dots per inch.

2.9.1.1 Measurement of dye permeation

The maximum of dye permeation was measured as the maximum distance the dye or ink travelled into cements from the point of contact with dye (i.e. the outer surface of the cement) to the point the dye had stopped permeating through the cement where dye or ink was no longer visible in the cement, illustrated in figure 4.

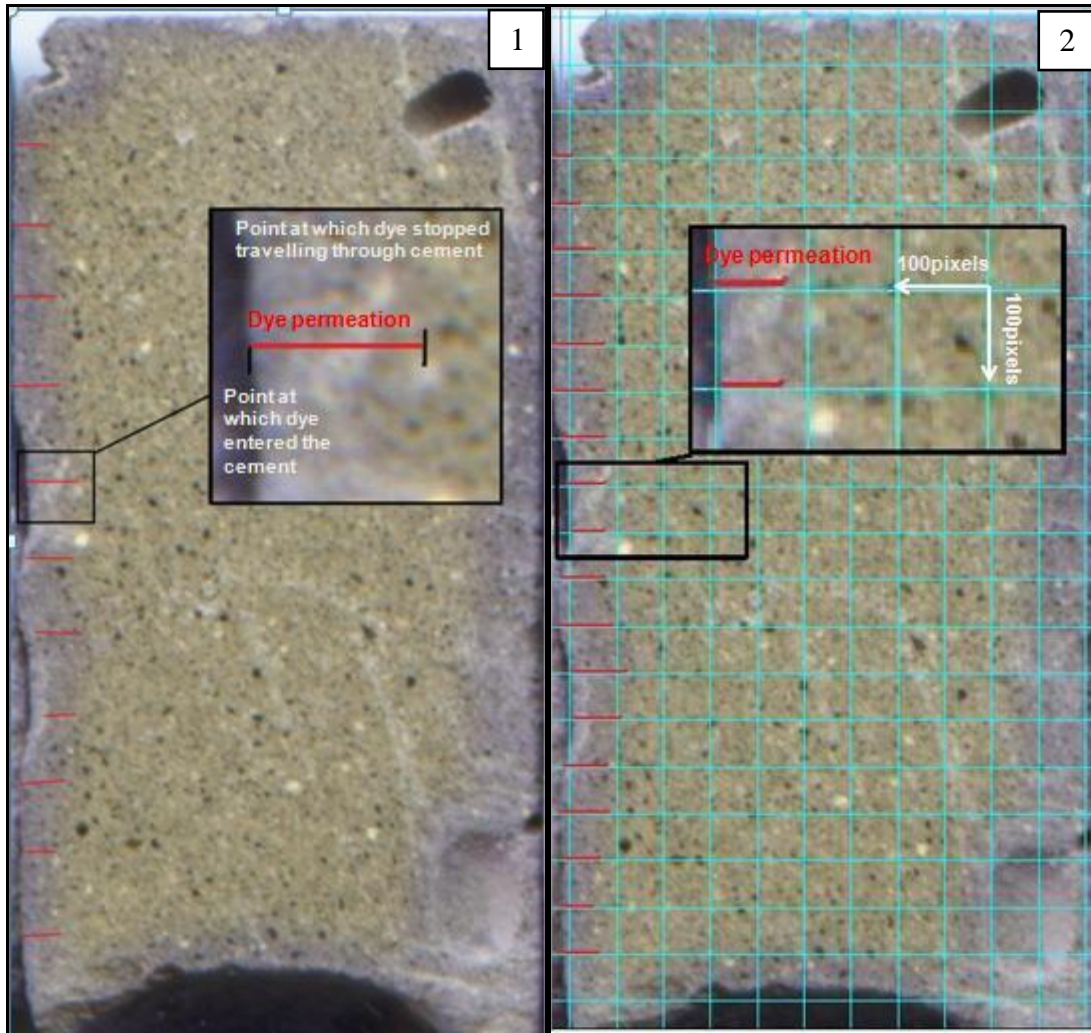


Figure 4: Images representing how dye or ink permeation through cement was measured using two methods, the first (1) representing the method used initially by taking randomly spaced measurements. For reliability of the measurements, an improved method (second method) was developed that involved taking measurements at set points. These points were determined by the presence of gridlines placed superficially on the image as shown in the second image (2).

Depths of dye permeation of cements were measured with Image J (Wayne Rasband, National Institutes of Health, USA) using the following methods.

The unit of length used to measure the movement of dye into cements was changed from inches to pixels using the image J software. The measurements taken were converted to millimetres by dividing the measurements taken (unit: pixels) by the value obtained for the

MATERIALS AND METHODS

number of pixels measured in one millimetre. The calibration of pixels to millimetres was undertaken by measuring the number of pixels between 0 and 1 mm on an image of a stage micrometer (Agar Scientific, England, UK), captured using a scanner (Epson Perfection V200 Photo, Epson Ltd, UK).

The first method for measuring dye permeation involved drawing a line from the point at which dye had entered a sample to the point where dye could no longer be observed in the sample. The improved method involved the use of a grid that lay superficial on the image of a sample that contained horizontal and vertical gridlines spaced 100pixels apart, equal to 0.5 mm (10,000pixels²). The gridlines were used as markers for where measurements should be taken as demonstrated in figure 4, image (1). Measurements were taken by drawing lines upon and along the gridlines, from the edge of the cement where dye had been seen to enter the sample, to the point where the permeation of the dye stopped. Measurements were repeated to obtain at least 15 values to produce the average measurement of dye permeation (in pixels) that was later converted to mm. Measurements of dye permeation with the aid of a grid enabled the production of more objective results, since the user took 15 measurements that were equally spaced apart and as such may provide a more reliable average result for average dye permeation. The depth of dye permeation was measured vertically from the longest sides of the cement sample for the majority of the study and also measured horizontally when focusing on the effect of sample preparation on the depth of dye permeation in cements.

2.9.2 Effect of dye permeation on PLR and cement composition

To obtain an overview of the effect of changing the PLR of model cement on dye permeation, a study was performed where cements at a PLR of 2.5, 3.3 or 4.0 g/ml were produced as described in previous methods and stored in dye for 3 days after 1, 10 and 30 days of

MATERIALS AND METHODS

setting prior to dye immersion. The effect of PoP addition (10 and 30 wt%) on dye permeation was also investigated and cement samples were stored in dye for 3 days after 1, 10 and 30 days of setting. These studies were carried out to see the effect of porosity (caused from varying the PLR and PoP addition) and PoP addition on the amount of dye permeation into the material, which could potentially give an indication of the sealing ability of the material in a moist environment.

2.9.3 Tooth model for dye leakage studies

Non-carious adult anterior teeth (premolars, canines and incisors) were used for this study (7teeth per cement variation investigated). Freshly extracted teeth were stored at -20°C and fixed with 10% formalin for 1 day and washed thoroughly before use. Straight-line access was used to gain access to the root canal since the technique provides an unimpeded pathway to the root canal [La Turno *et al*, 1985]. Straight-line access was achieved by producing a large access cavity using a high speed handpiece with either a 541 or 501 M HiDi diamond bur (Densply Ash instruments). Roots were accessed via the crown by cutting down through the dentine and pulp tissue until the root canal opening was visible (see figure 5). The step-back method involved the use of endodontic files to produce a flared and tapered preparation of the root canal, beginning with the largest apical file (size 40) and followed by incremental shortening of the files to create the appropriate shape for the root canal [Torabinejad *et al*, 2002]. Endodontic nickel titanium files (K Files, Kerr, Italy) were inserted into the root canal to locate the pathway of the root and to clear the root canal of pulp debris using the step-back method.

A low speed handpiece containing a Gates Glidden bur (size 3) was used to relocate the path of the root canal and slightly widened the root canal. A standard diameter and length of the root canal preparations was essential for consistency in root canal preparation. A portable laboratory handpiece/motor set at 300rpm (KaVo Ltd, UK) containing a flat ended 542-fissure

MATERIALS AND METHODS

bur was used to create a cavity with a standard diameter of 1 mm throughout (see figure 5) that followed the pathway of the root canal, produced using the Gates Glidden bur previously. A diameter of 1 mm was selected for clinical relevance despite the literature stating that 1 mm thick MTA root fillings were less effective at preventing coronal leakage of India ink than thicker MTA root filling preparations [Ölmez *et al*, 2008].

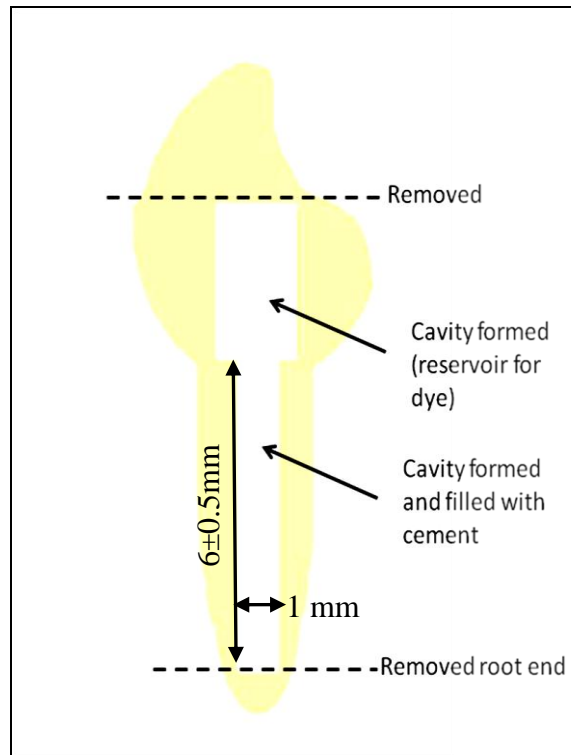


Figure 5: Image representing a tooth prepared to be root filled with cement.

The apices of the teeth were removed using a 501 M HiDi diamond bur (Densply Ash instruments) since the tapered ends were not required (see figure 5). Root cavity length of 6 ± 0.5 mm was determined using the length of an endodontic file with a rubber stop. A cavity length of 4 mm was determined to be significantly more effective than <3 mm [Valois *et al*, 2004] therefore the length used for the study was 6 ± 0.5 mm (see figure 5). The rubber stop on an endodontic file was used to mark the openings of the root canal and measured using a digital

MATERIALS AND METHODS

Vernier to determine the full length of the root canal. The required length was deducted from the value measured for the length of the root canal using the file and the calculated length to be deducted from the length of the tooth by making markings onto the tooth with a permanent marker. The length to be removed to achieve a standard length of 6 ± 0.5 mm was removed using a 501 M HiDi diamond bur (Densply Ash instruments).

Roots were filled with either 100 wt% MTA, 80 wt% MC 20 wt% Bi_2O_3 , 90 wt% MTA or 70 wt% MC 20 wt% Bi_2O_3 containing 10 wt% PoP. 20 μl of 5 % Crystal violet was placed in the crown portion of the tooth, which acted as a reservoir for the dye, see figure 5. The teeth were kept upright for storage in 96-well culture plates, aided using a little sticky wax and placed in a humidified incubator (at 37°C) for 3 days. Dye was replenished on a daily basis to eliminate the effects of dye being lost through evaporation.

Dye was removed using tissue to dry the crown portion holding the dye. Teeth were sliced longitudinally using an Isomet low speed saw (Buehler, Illinois, USA) with a diamond blade (MetPrep Ltd, Coventry, UK) and air dried for 3 days. Surfaces exposed were polished by hand using silicon carbide paper as described previously to produce a flat surface in order to enhance image quality as this evenly distributed the surface roughness of the sample, so that the surface of the sample appeared to be on one plane when the image was captured. Images of the halves were taken using a scanner (Epson Perfection V200 Photo, Epson Ltd, UK) and dye permeation and penetration into cements were recorded using Image J. Dye penetration was the measurement of the movement of dye through cement from point of contact with the tooth to the point at which no more dye was visible in the cement.

2.10 *Statistical analysis of raw data*

Mean values were compared (using raw data) for significance testing ($p < 0.05$) using a one-way ANOVA with Tukey post-hoc test (PASW Statistics 18.0 for Windows, SPSS Inc, USA).

RESULTS

3 Characterisation and generation of a model system

3.1 SEM evaluation of cement powders

Cement powders were qualitatively analysed in terms of particle size and distribution. Despite sieving (section 2.1.2), OPC and to a lesser extent, SC, contained agglomerate particles which were evident at x 200 magnification (images were labelled macrostructure since the images were captured using a low magnification with the SEM) as seen in the photomicrographs in figure 6. SC powder contained fewer agglomerates that were $>20\mu\text{m}$ in diameter compared with OPC and MC and also contained smaller particles than observed in OPC powder ($>30\mu\text{m}$). MC powder consisted of particles that were relatively homogenous in size, observed by looking at the distribution of particles in SEM micrographs of the macrostructure in figure 6, with the smallest particles $<15\mu\text{m}$ in diameter (see figure 6, microstructural images). SC and OPC contained a combination of relatively small ($<15\mu\text{m}$) and larger ($20\text{-}30\mu\text{m}$) particles as seen in the SEM micrographs in figure 6.

RESULTS

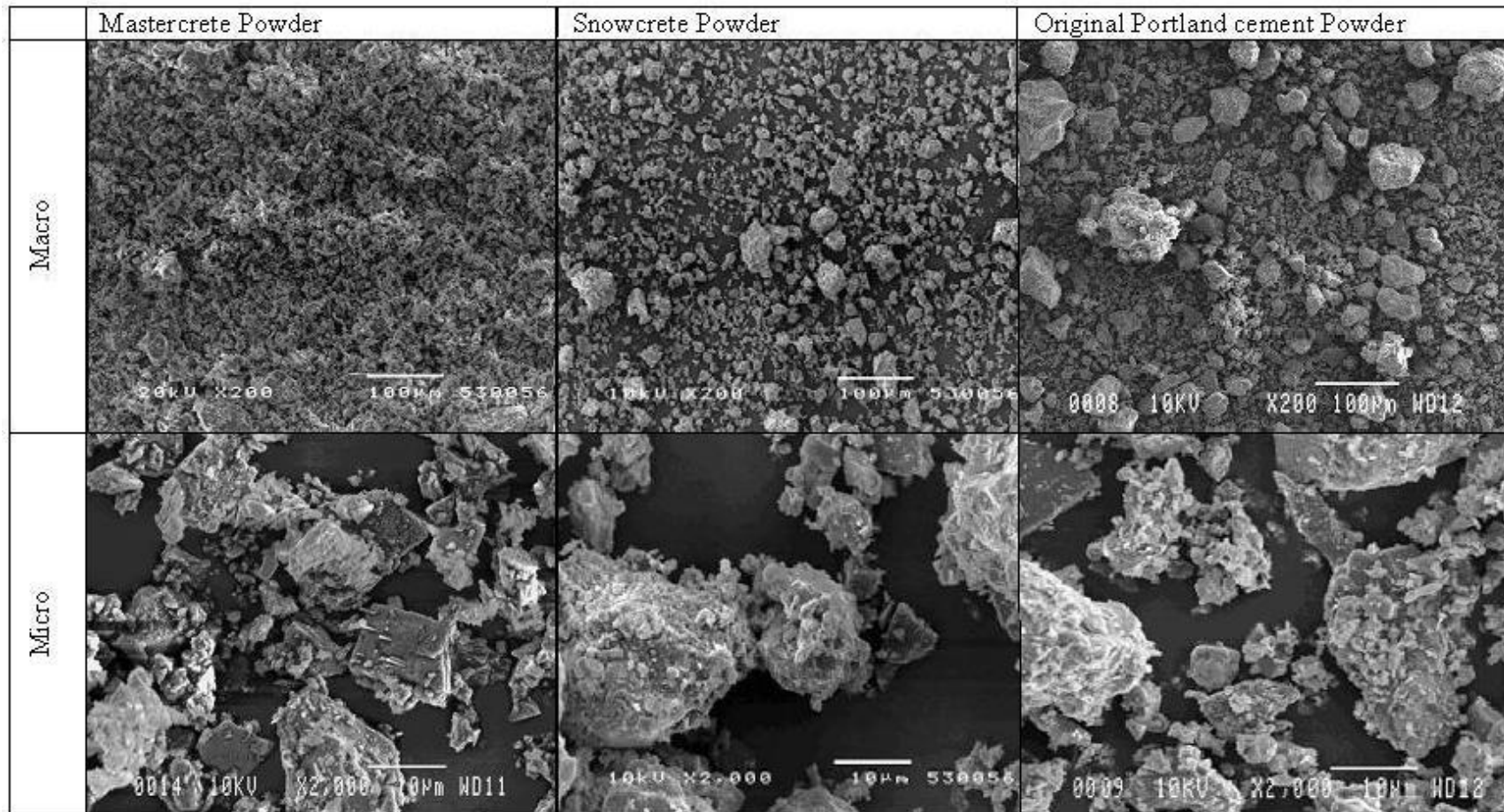


Figure 6: Scanning electron microscope (SEM) photomicrographs of Portland cement powders. Mastercrete particles were smaller than Snowcrete, which in turn were smaller than Original Portland cement. MC had the most homogeneously-sized particles.

3.2 CS and RP of pure cements

CS decreased with increased PLR for 100 wt% MC but CS increased with increased PLR for 100 wt% SC and OPC, as shown in figure 7. 100 wt% MC with a PLR of 2.5 g/ml had a significantly higher CS (67.1 ± 16.6 MPa) when compared with other cements with the same or higher PLR ($p < 0.05$). 100 wt% MC was the only cement found to be workable at PLR 4.0 g/ml compared with SC and OPC at this PLR, which were unworkable.

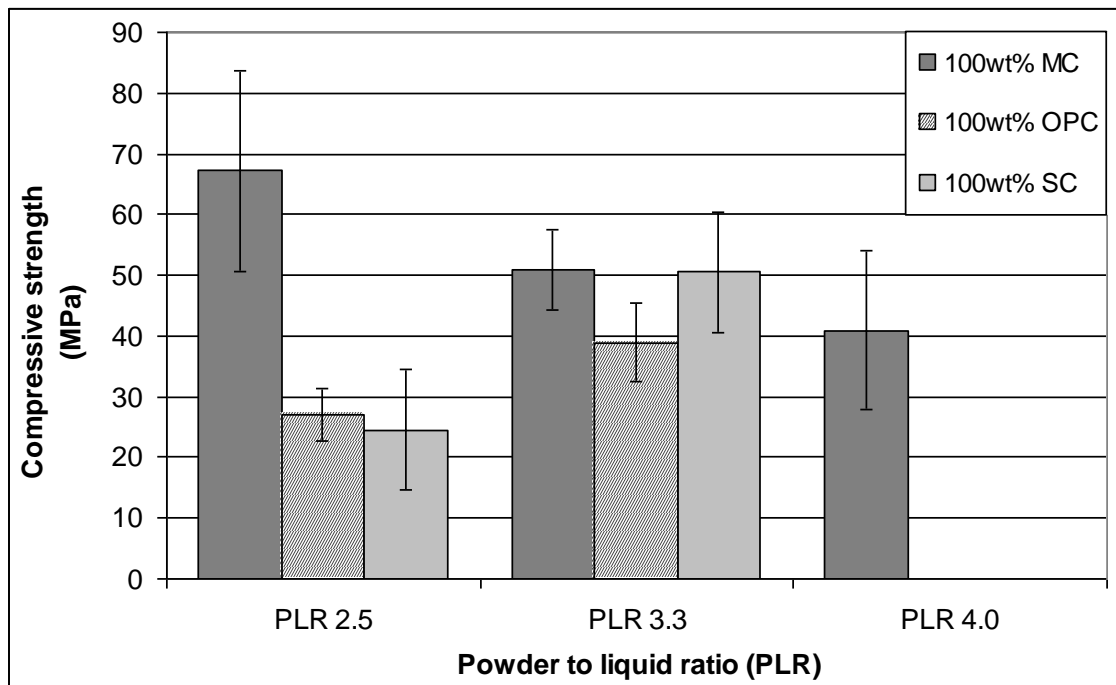


Figure 7: Compressive strength (CS) of PCs at various PLR. 100 wt% MC generally had a higher CS than other cement types and MC was the only cement which was workable at a PLR of 4.0 g/ml.

The RP of 100 wt% MC, OPC and SC at a PLR 2.5 g/ml was similar as shown in figure 8 and RP decreased with increased PLR (see figure 8).

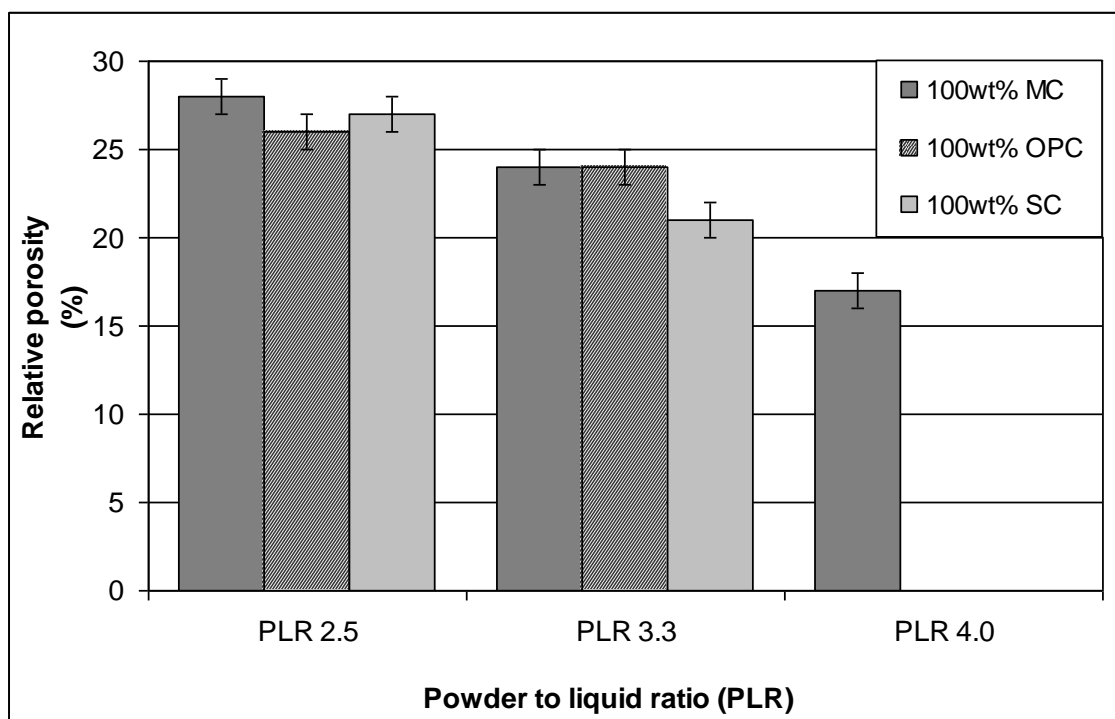


Figure 8: Relative porosity (RP) of Portland cements at various PLR. Little difference was observed for cements that had the same PLR. The RP generally decreased with increasing PLR. Error bars represent the approximate error of the method and calculations used to retrieve RP ($\pm 1\%$).

Based on the results observed in this chapter (discussed later on in this thesis), the PLR used for subsequent studies (unless otherwise stated) was 3.3 g/ml.

3.3 *Selection of the bismuth oxide component of the model system*

Little difference was observed between the two Bi_2O_3 powders, examined using a SEM, although it was observed that Bi_2O_3 Acros powder had apparently more particles larger than ($>5\ \mu\text{m}$) than those examined for Bi_2O_3 Sigma (figure 9).

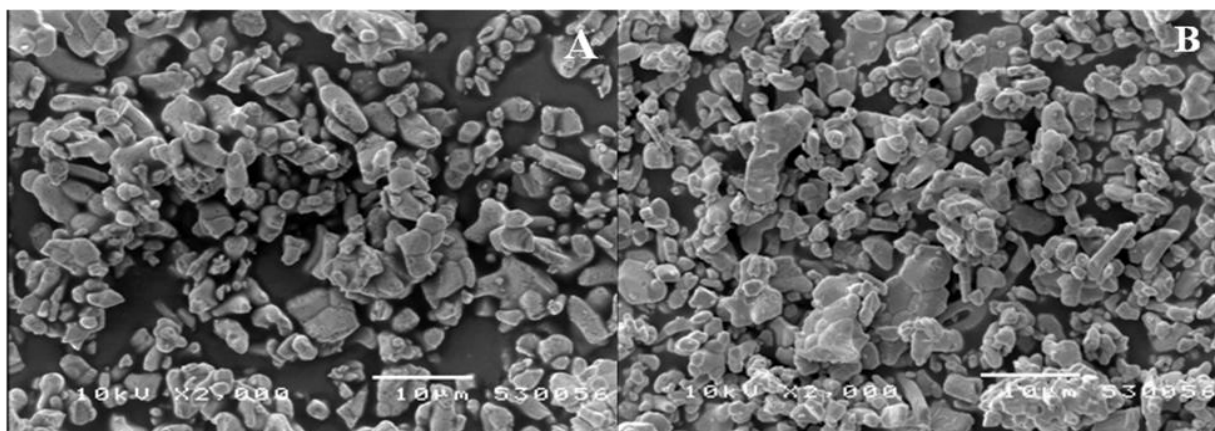


Figure 9: SEM photomicrographs of two types of Bi_2O_3 powder from the manufacturers (A) Acros Organics (Acros) and (B) Sigma Aldrich.

Cements containing Bi_2O_3 Sigma had a slightly higher CS when compared with samples containing Bi_2O_3 Acros (see appendix 1 for values) but this was not significant ($p>0.05$). Both cements containing different Bi_2O_3 had an RP of $20 \pm 1\%$. SEM examination showed little differences in the macro and microstructure of the set cements, as shown in figure 10.

Following the analysis of the results from the studies highlighted in this section of the thesis, Bi_2O_3 Sigma was selected for use as a component of the model cement.

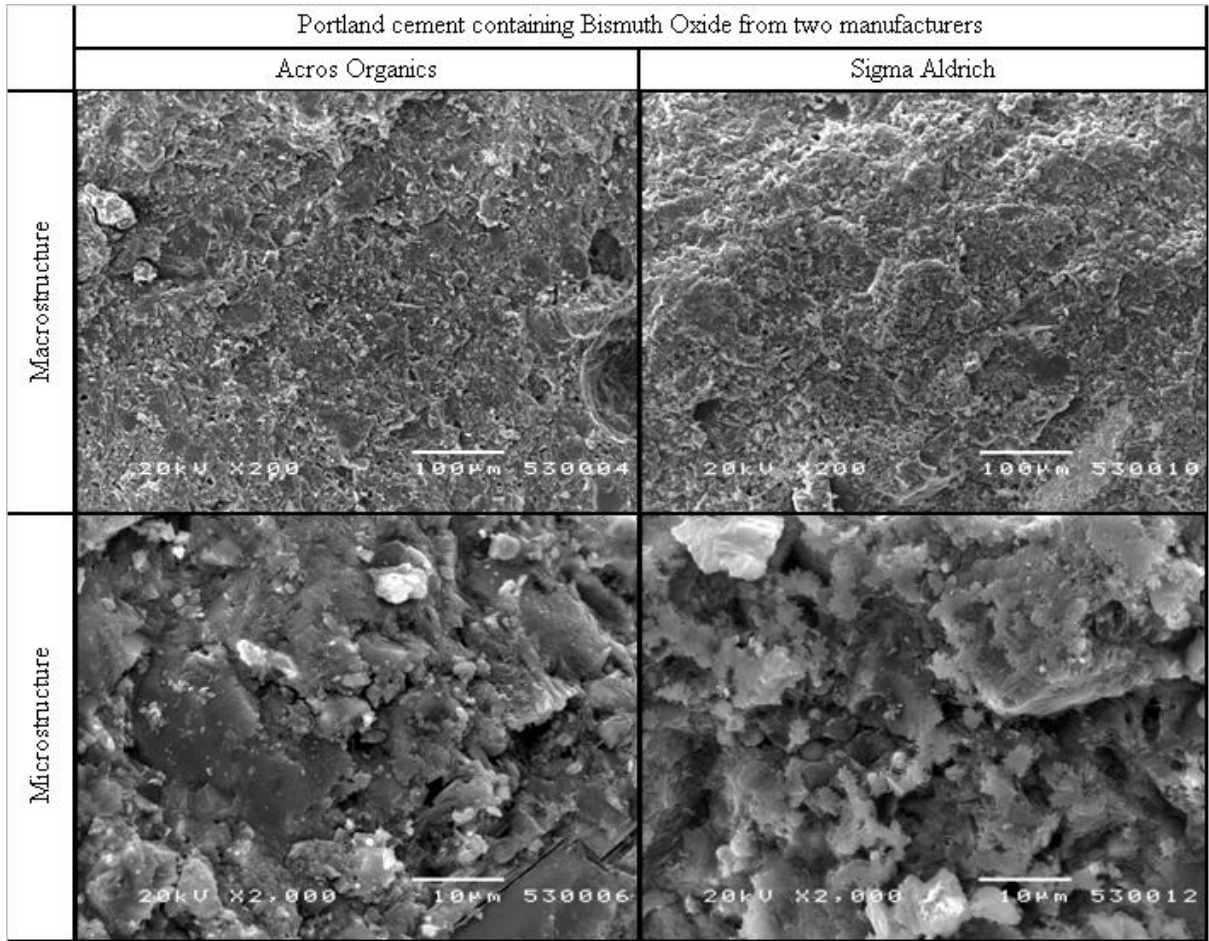


Figure 10: Scanning electron micrographs of cements containing Bi_2O_3 from different manufacturers. The macro and microstructures appeared to be very similar regardless of the type of Bi_2O_3 added to the cements.

3.4 *Effect of mould storage time on stability and structure of cement*

Samples that were placed into distilled water after various time points had similar CS since no significant differences were observed (figure 11). Model cements set for 1 and 3 days before water immersion had the lowest RP ($20 \pm 1\%$) compared with cements stored for 6 h and 7 days before water immersion, as shown in figure 12. Samples stored after 7 days of setting had the highest RP ($27 \pm 1\%$) compared with other storage times, which was shown in the bar chart in figure 12. The trend suggests that long storage time (7 days) meant that the CS decreased and RP increased (figures 11 and 12).

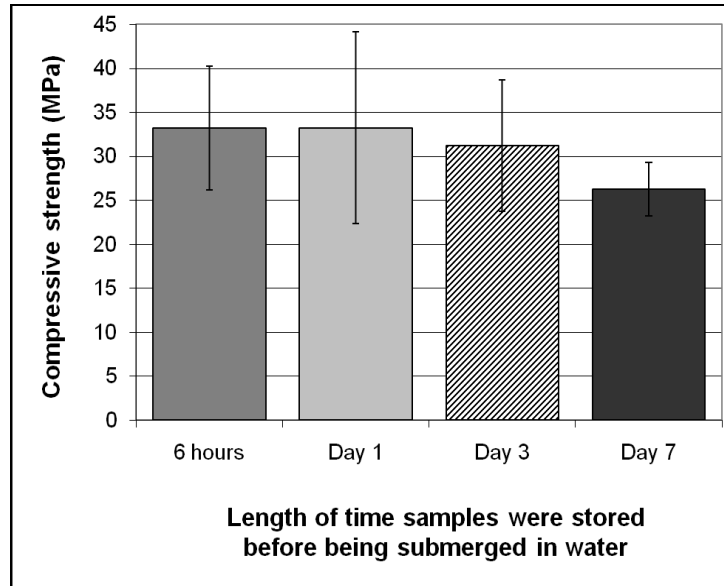


Figure 11: CS of the model cement set for various lengths of time before water storage. CS decreased when the model cement was immersed in water after setting for 7 days when compared with cements set for shorter time periods. Error bars represent the standard deviations for the mean data plotted in the histogram.

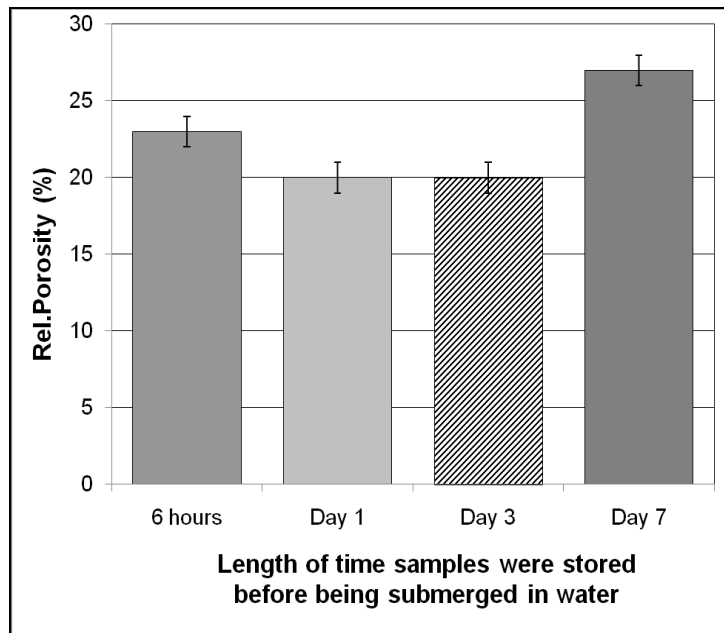


Figure 12: RP of model cements that were set for different time periods before water storage. Error bars represent the total error of the method and calculations used to retrieve RP ($\pm 1\%$). RP increased when cements were stored for 7 days but remained low with shorter setting times before water immersion.

RESULTS

SEM examination (figure 13) of the cement fragments revealed that there were considerable differences in the microstructure of the outer surface of these samples.

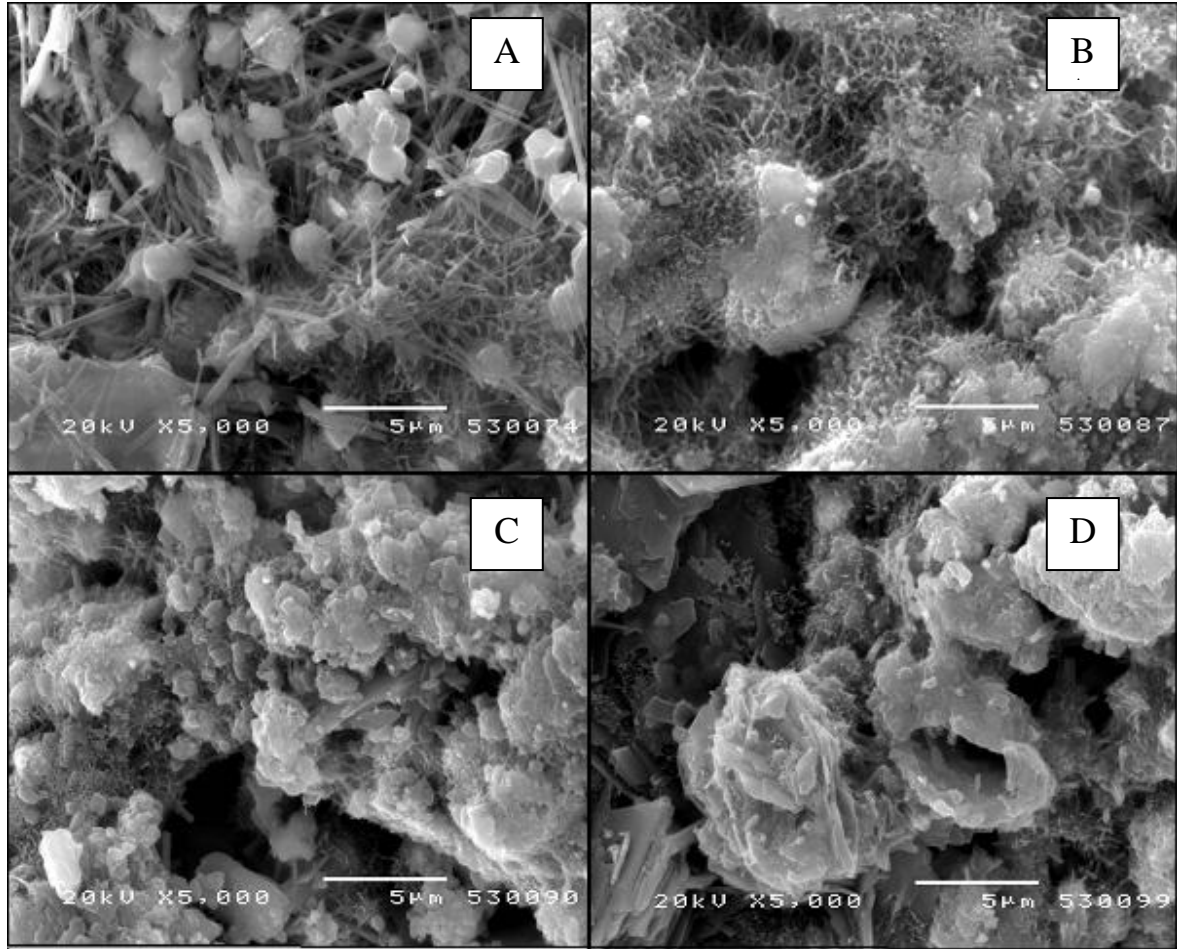


Figure 13: SEM photomicrographs of the outer surface of cement samples. Samples were placed into water at different time periods. Image A. Cement placed into water after 6 hours of setting; Image B. after 1 day of setting; Image C. after 3 days of setting and Image D. after 7 days of setting.

Samples stored for 3 and 7 days did not show the characteristic needle-like structures present on the outer surface of cements set for 6 h and 1 day. Crystals seen in samples stored for 3 and 7 days were large and plate-like, which contrasted with the fine needles seen in samples stored for 6 h and 1 day (figure 13).

RESULTS

Cement samples were stored for 1 day before extraction from the mould for further storage in solutions for subsequent studies (unless otherwise stated).

4 Incorporation of additives to the model cement and MTA

4.1 *Microstructure of calcium sulphate powders*

Additives were examined using SEM to determine the shape and size of particles to enable comparison with structures seen in the set cement. SEM examination revealed PoP contained particles that varied in size (between 5-20 μm) and shape (figure 14). Some of the particles appeared to be similar in size and shape when compared with anhydrous calcium sulphate particles (CaSO_4) from Sigma Aldrich (figure 15).

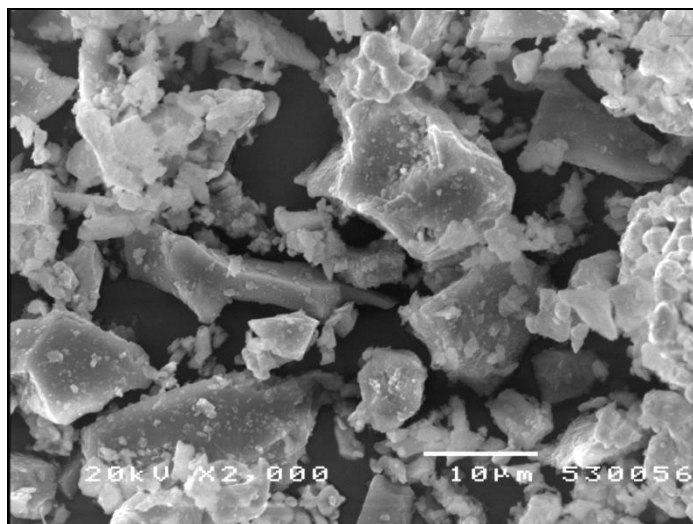


Figure 14: SEM photomicrograph of Plaster of Paris (PoP) powder showing heterogeneously sized particles.

Gypsum (Gyp) from Acros Organics appeared to have similar sized particles ($>10\mu\text{m}$ in length) in comparison with PoP and Gyp (Sigma), however, Gyp differed in shape with elongated and bean-shaped particles (figure 15) when compared with PoP. CaSO_4 (Acros) consisted of heterogeneously sized particles, containing large particles $>30\mu\text{m}$ in length whereas CaSO_4 (Sigma) had particles that were much smaller and appeared to be more homogeneous in size,

>10 μ m (figure 15). Some of the particles observed in CaSO₄ (Acros) appeared to be similar to those seen for Gyp (Sigma and Acros), shown in figure 15.

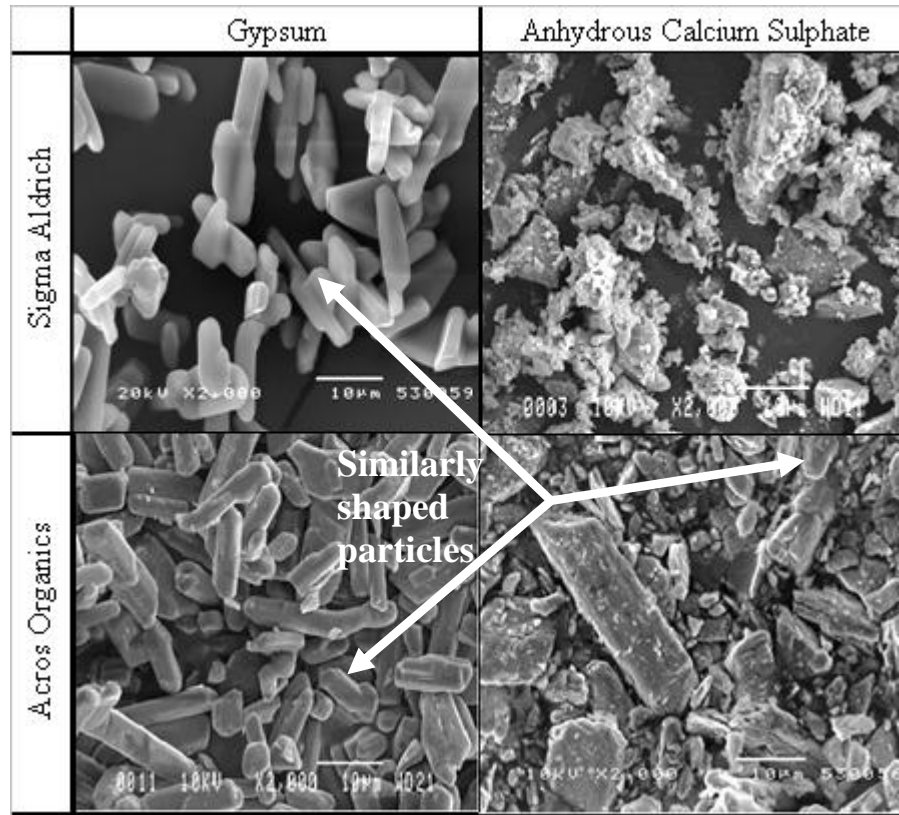


Figure 15: SEM photomicrographs of additive powders. Large differences were found between calcium sulphates, particularly CaSO₄ from different manufacturers as larger particles were observed in Acros Organics CaSO₄ when compared with particles in Sigma Aldrich CaSO₄.

4.2 *Modifying the model cement with calcium sulphates*

MC-based model cements (the control) and cements containing gypsum (Sigma and Acros) did not set within the investigated time period of 6 h at room temperature. The setting time decreased considerably when calcium sulphates, PoP and CaSO₄ were incorporated into the cements and the setting times decreased the most with larger additions of PoP and CaSO₄ (as shown in figure 16). 20 wt% CaSO₄ (Sigma) and PoP containing cements had the lowest initial

RESULTS

setting times of all cements. Cements containing CaSO_4 (Sigma) always had the lowest setting times when compared with CaSO_4 (Acros)-containing cement (figure 16).

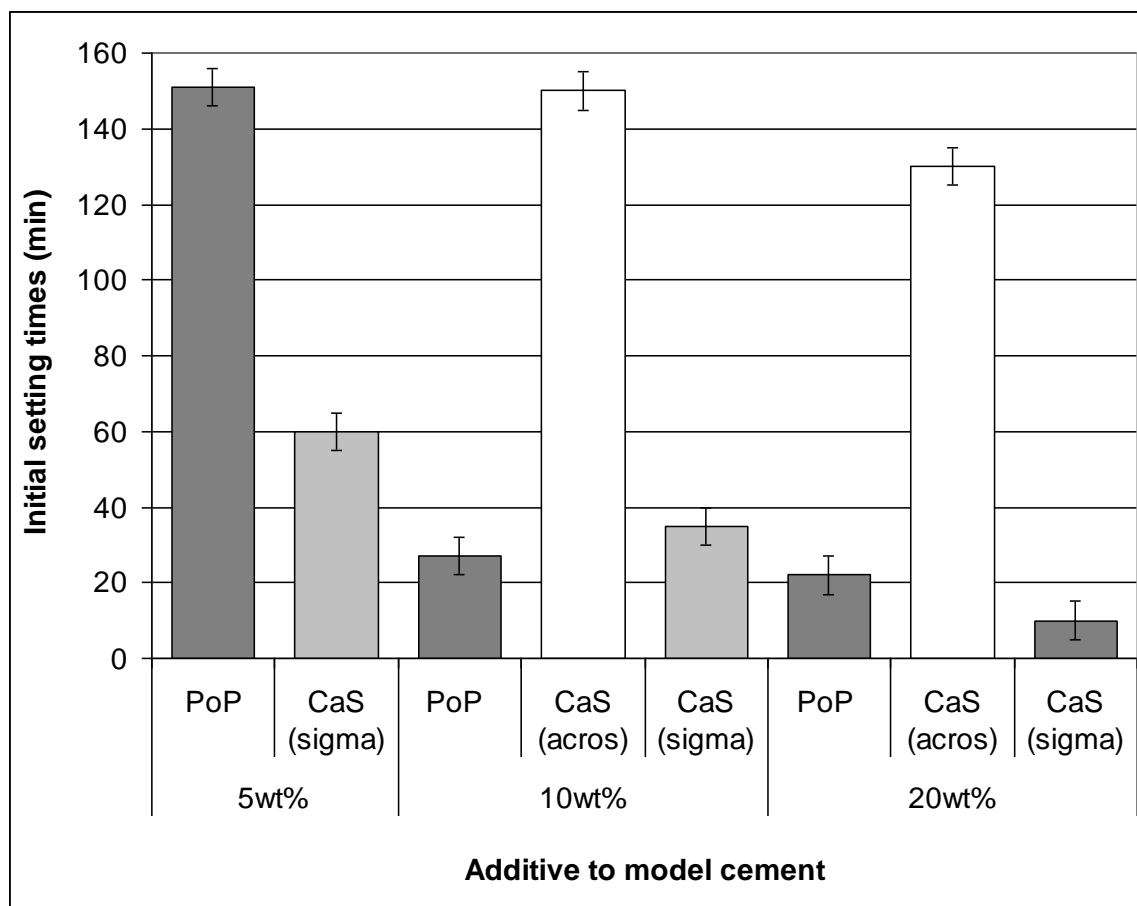


Figure 16: Initial setting times of the model cement containing the different calcium sulphates at various wt%. Initial setting times decreased considerably with increased additions of calcium sulphates with the most dramatic decrease in setting time observed when adding PoP or CaSO_4 (Sigma) to cement. Error bars represent the total error of the method (± 5 min).

Small additions (5 wt%) of calcium sulphates, PoP and CaSO_4 (Sigma and Acros) to the model cement gave rise to a significantly higher CS compared with the model cement only (see figure 17) but these additions had little effect on the RP (see figure 18).

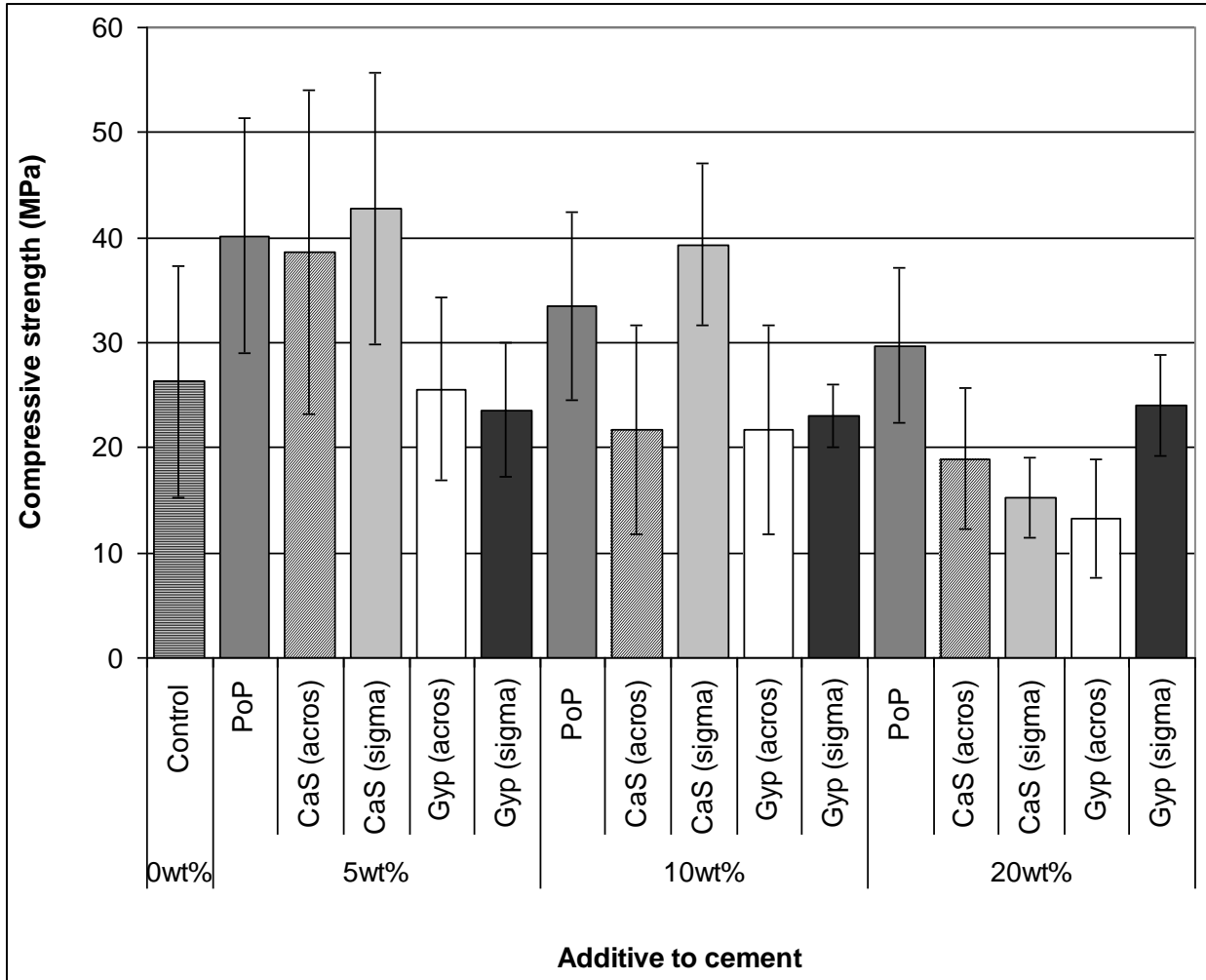


Figure 17: CS of the model cement containing various calcium sulphate additives. PoP-containing cements always had a higher CS than the control. Large additions of calcium sulphates to MC generally led to a decrease in CS. Error bars represent the standard deviations of the mean results plotted on the graph.

10 wt% CaSO_4 (Acros)-containing cement had a lower CS than the control (but this was not significant, $p > 0.05$). Despite the difference observed in CS of 10 wt% CaSO_4 (Acros)-containing cement and cement with no additions, the RP remained the same. In comparison with 5 wt% additions, 10 wt% PoP and CaSO_4 (Sigma) additions to the cement continued to demonstrate higher CS than cements with other additives and these CS were not statistically different to each other (figure 17).

RESULTS

Cements containing 20 wt% CaSO_4 (Acros and Sigma) had significantly lower ($p < 0.05$) CS when compared with the model cement containing 5, 10 or 20 wt% PoP and 5 or 10 wt% CaSO_4 (Sigma or Acros), however, the low CS was not significantly different ($p > 0.05$) to the model cement without calcium sulphate additions and cements containing Gyp (Sigma or Acros).

RP increased with 20 wt% additions of CaSO_4 (Sigma and Acros) to 28 ± 1 and 30 ± 1 % respectively and was considerably higher than the model cement and cement containing other additives at 20 wt% (figure 18). Cements containing CaSO_4 (Sigma) generally had higher CS and lower RP than cements containing CaSO_4 (Acros) though significant differences were only observed at 20 wt% additions ($p < 0.05$).

Gyp-containing cements had lower CS than cements containing CaSO_4 or PoP but Gyp-containing cements but these differences were not significant ($p > 0.05$) when compared with the control (model cement), shown in figure 17. Cements containing Gyp always showed the highest RP at any given addition (i.e. 5, 10 and 20 wt%), see figure 18.

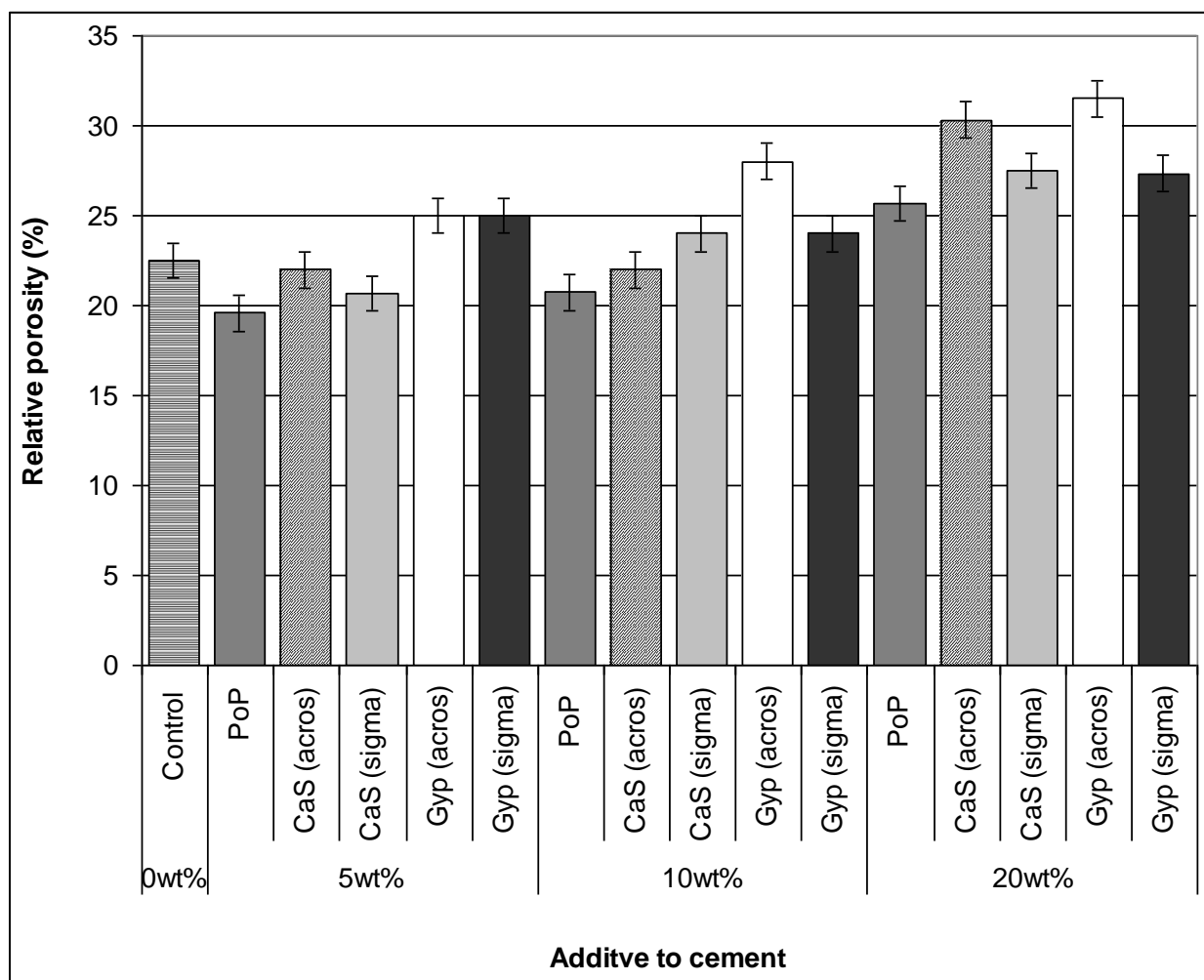


Figure 18: RP of the model cement containing various calcium sulphate additives. Gyp-containing cements showed the highest RP for all levels of additions. Small additions (5 wt%) of calcium sulphates PoP and CaSO₄ (Sigma and Acros) had little effect on the RP of cement. Large additions of these calcium sulphates increased the RP of cements. Error bars represent the total error of the method and calculations used to retrieve RP ($\pm 1\%$).

CaSO₄ (Sigma) and Gyp (Sigma) were selected as opposed to CaSO₄ (Acros) and Gyp (Acros), for incorporation into model cements and MTA to carry out further studies.

4.3 *Varying additive concentrations in the MC model cement*

4.3.1 **Effect of additives on initial setting times of model cement**

The control consisting only of MC and Bi_2O_3 , and Gyp-containing cements did not set within the investigated time period (>6 h). Additions of PoP or CaSO_4 decreased the initial setting time of the control (figure 19) and with increased PoP or CaSO_4 content, the initial setting time decreased considerably by up to 90 % as shown in figure 19. 5 wt% additions of PoP or CaSO_4 to cement decreased the initial setting times of cements by approximately 58 % and 80 % respectively, compared with the control. 5 wt% CaSO_4 additions had a more profound effect on the initial setting time of the control than the addition of 5 wt% PoP, initially setting in about one third of the time that it took for 5 wt% PoP-containing cements to set (figure 19).

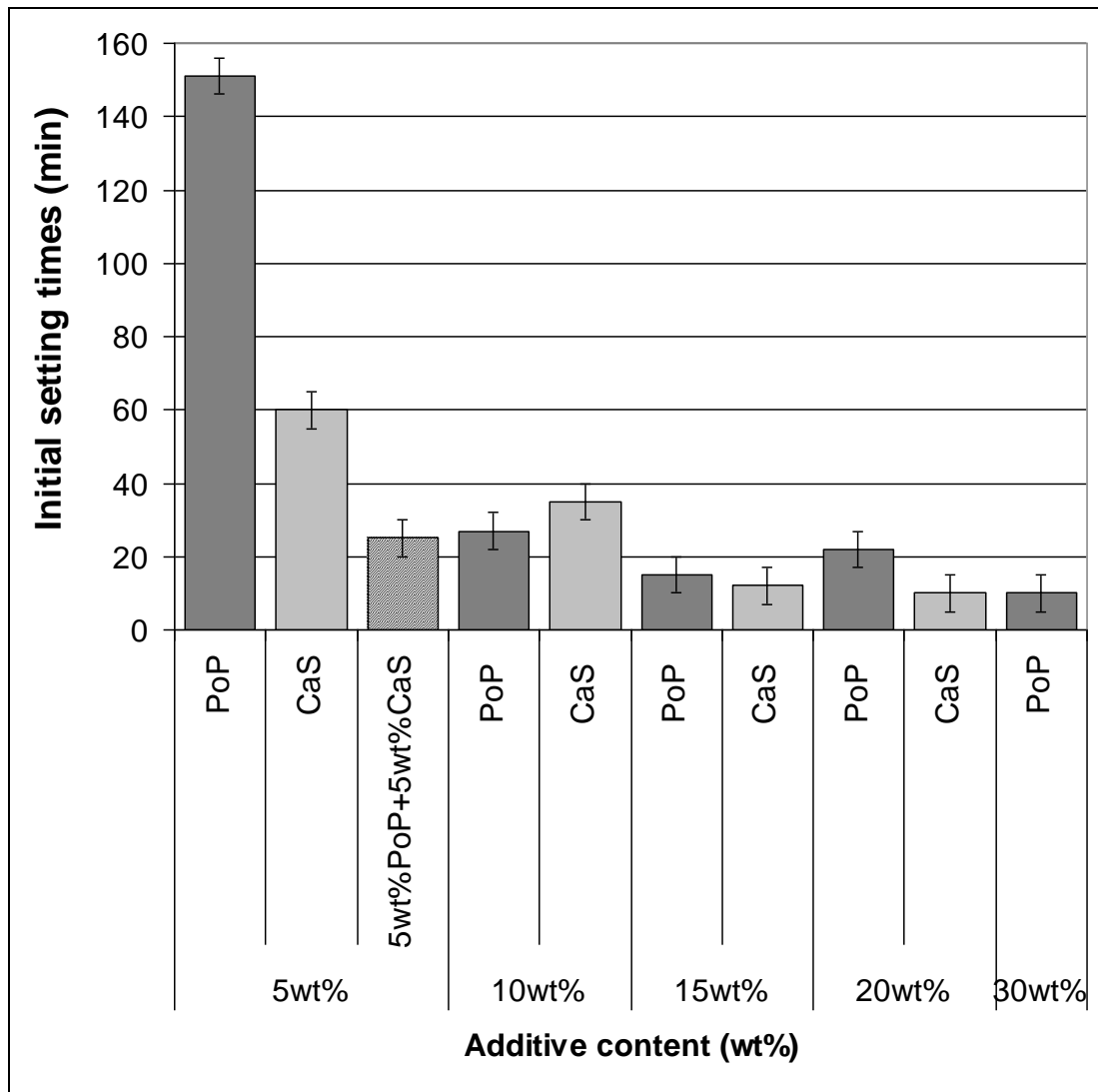


Figure 19: Initial setting times for the model cement containing calcium sulphates. Model cements with no additions of calcium sulphates and Gyp-containing cements did not set in the investigated time period. Increasing the amount of calcium sulphates additions, CaSO_4 and PoP decreased the setting times the most. Error bars represent the total error of the method (± 5 min).

There was a considerable difference in the initial setting times of 5 and 10 wt% PoP, as 10 wt% PoP-containing cements setting up to five times faster than cements containing 5 wt% PoP. 10 wt% CaSO_4 addition to cement decreased the setting time by nearly one half (figure 19) when compared with the addition of 5 wt% CaSO_4 . 5 wt% PoP + 5 wt% CaSO_4 -containing cements decreased the initial setting time more than 10 wt% PoP or 10 wt% CaSO_4 -containing cements as

5 wt% PoP + 5 wt% CaSO₄-containing cements had a slightly lower setting time. Addition of 15 wt% PoP and CaSO₄ decreased the initial setting times of cement by up to one half when compared with the effect of 10 wt% PoP and CaSO₄ respectively. Little difference was observed between the initial setting times of cements with 15 wt% or 20 wt% additions of PoP and CaSO₄ respectively (figure 19).

4.3.2 CS and RP of model cements containing calcium sulphates

5 wt% PoP and CaSO₄ additions to cements had the highest CS and these were significantly different to the model cement without PoP or CaSO₄ additions ($p < 0.05$). Increased addition of PoP and CaSO₄ (10 wt%) caused the same effect on the CS of cement, since the CS were 30 % greater than the control (see figure 20) but these differences were only significant with 10 wt% PoP additions ($p < 0.05$). The RP remained similar to the model cement for cements containing 5 wt% CaSO₄ additions and was slightly lower than the control for 5 and 10 wt% additions of PoP to the model cement. The RP increased (24 ± 1 %) for 10 wt% CaSO₄-containing cement when compared with smaller additions of CaSO₄ to the model system (see figure 21).

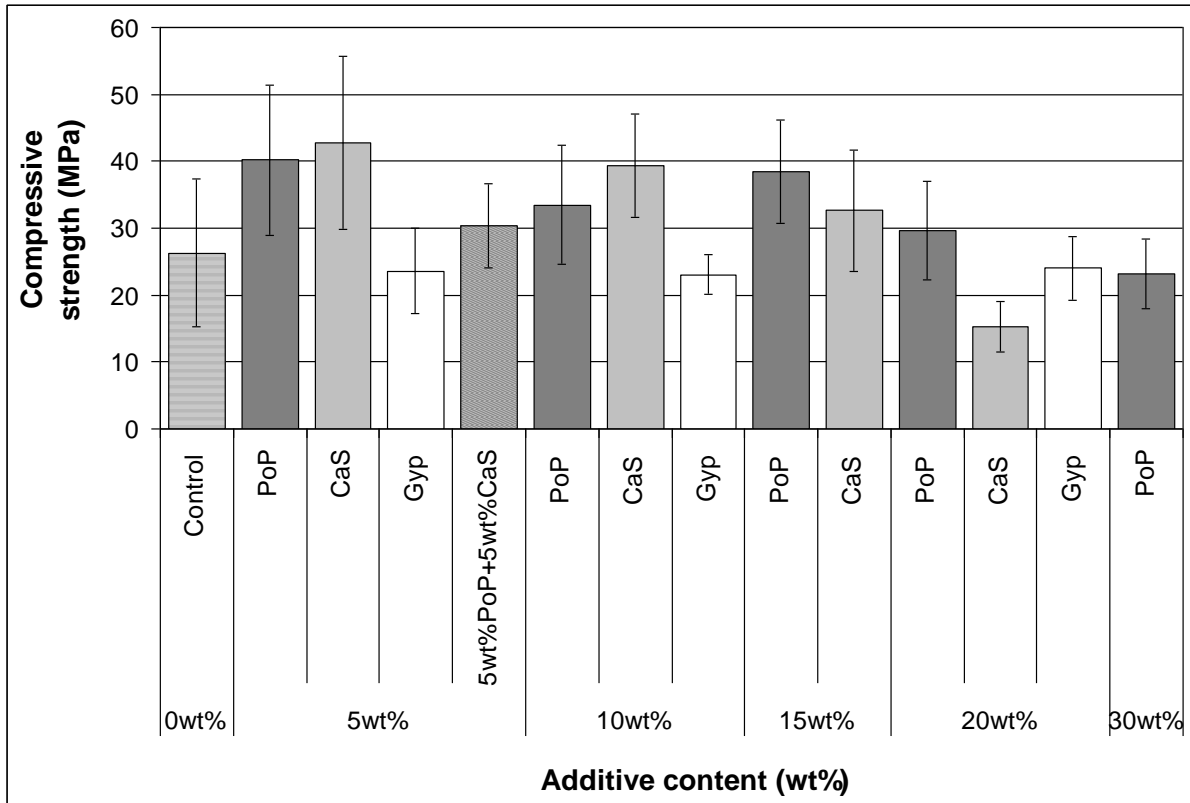


Figure 20: CS of model cements containing 5 to 30 wt% additions of calcium sulphates. 5 and 10 wt% PoP and CaSO_4 additions increased the CS of cements; however, increased addition (20 and 30 wt%) of PoP and CaSO_4 decreases CS. Error bars represent the standard deviations for each mean value plotted.

Combining 5 wt% PoP additions with 5 wt% CaSO_4 additions to cement had a similar effect to 10 wt% PoP or CaSO_4 additions since the CS was considerably higher than the control, though the higher CS was not statistically significant ($p > 0.05$). However, the CS was much lower for combined additions of 5 wt% PoP and 5 wt% CaSO_4 when compared with 5, 10 and 15 wt% additions of PoP and CaSO_4 . The RP of cement containing 5 wt% PoP + 5 wt% CaSO_4 was similar to that of 10 wt% CaSO_4 and slightly higher than the RP of the model cement.

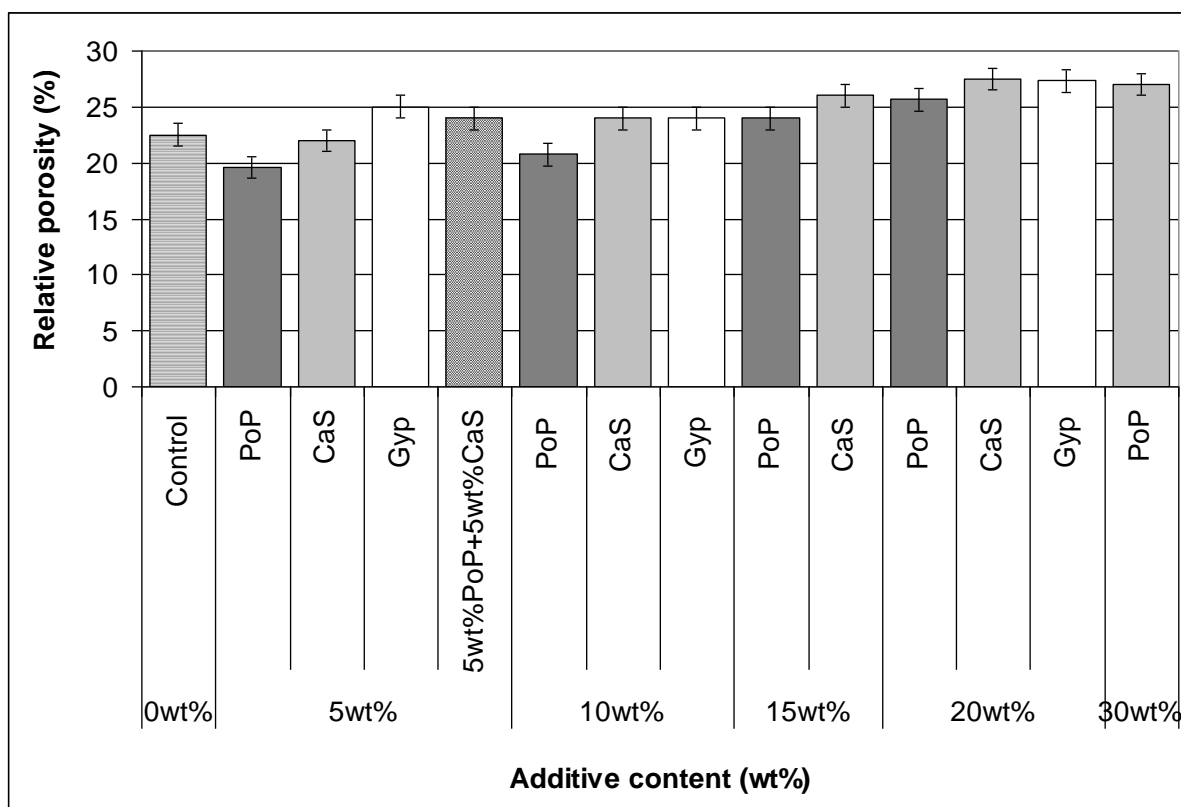


Figure 21: RP of model cements containing additives of various weight percentages (wt%). Small increases in RP were evident with the increasing PoP and CaSO_4 content. Large additions of calcium sulphates generally generated the highest RP in MC. Error bars represent the total error of the method and calculations used to retrieve RP ($\pm 1\%$).

Additions of 20 wt% calcium sulphates led to decreased CS and increased RP of cements when compared with the model cement and cements containing lower concentrations of calcium sulphates. 20 wt% CaSO_4 additions to the model cement generated the lowest CS, which was less than two thirds of the CS of the control, though the CS was not significantly different ($p>0.05$) to the CS of Gyp-containing cements and had one of the highest RP. Cement with 20 wt% PoP had a CS approximately double that of cement containing 20 wt% CaSO_4 but this was not statistically significant ($p>0.05$). Since there was a large difference in the CS and RP of 10 and 20 wt% CaSO_4 , 15 wt% CaSO_4 additions were incorporated with the model cement. 15 wt% PoP and CaSO_4 additions to cements did not significantly alter the CS compared with cements containing 5 and 10 wt% PoP and CaSO_4 , however, these additives had an effect on the RP which increased

to 24 ± 1 and 26 ± 1 %, respectively, when compared with the control and cements with low concentrations of PoP and CaSO_4 (figure 21).

Cements containing 30 wt% PoP had a CS that was significantly lower ($p < 0.05$) than the control and a higher RP compared with the RP of the model system (see figure 21). 20 wt% Gyp additions to the model cement generated similar CS to cement with 5 or 10 wt% additions of Gyp; however, cement with 20 wt% Gyp contained the greatest RP observed in this study (figure 21).

4.3.3 Examination of the set microstructure with SEM

4.3.3.1 *Fracture surface of cement samples*

The fracture surfaces of cement containing PoP were similar to the model cement when the microstructure was observed (defined as x 2000 magnification). Model cement without calcium sulphate additions and cements containing 5 wt% PoP had large plate-like crystals present, with some small particles upon these plate-like crystals (figure 22).

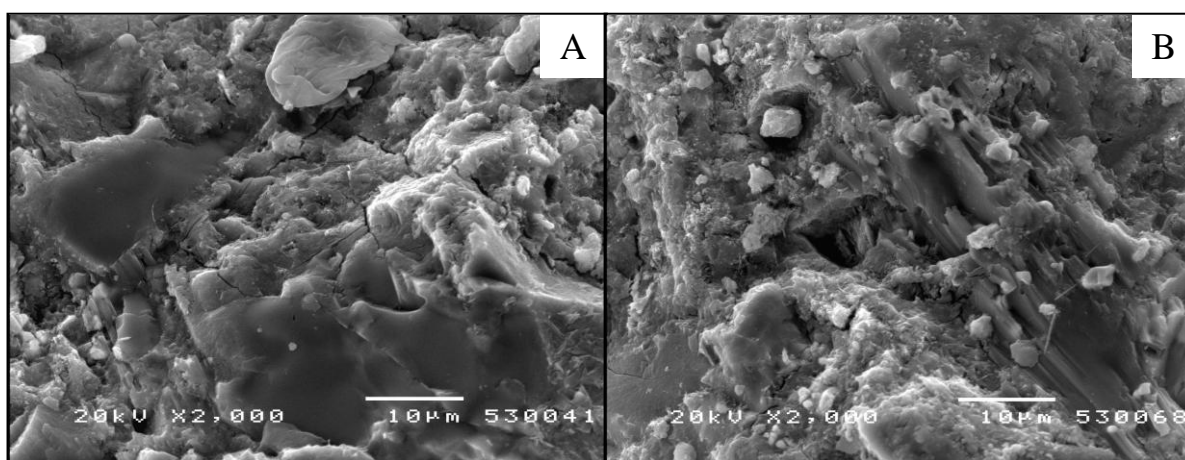


Figure 22: SEM photomicrographs of the fracture surface of A. the model cement and B. cement containing 5 wt% PoP. The structure of cement did not appear to differ with the addition of low concentrations of PoP to cement.

RESULTS

Cements containing 10 wt% PoP had smaller crystals when compared with 5 wt% PoP and the control. Elongated, rectangular-shaped particles were evident in MC containing 20 wt% PoP, resembling those in 20 wt% Gyp-containing cements (figure 23). There were no changes in the cement structure of 30 wt% PoP-containing cements when compared with 20 wt% PoP-containing cements observed using SEM.

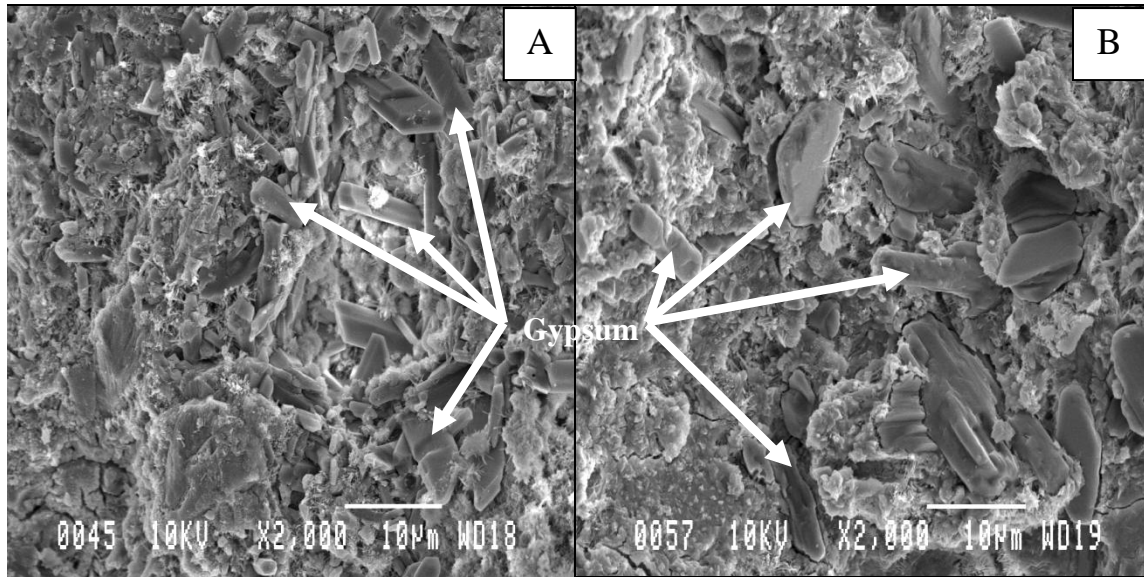


Figure 23: SEM photomicrographs of the fracture surface of cement samples containing A. 20 wt% PoP and B. 20 wt% Gyp. Gypsum particles (indicated by arrows) were seen in cements containing 20 wt% PoP and 20 wt% Gyp.

Observing the fracture surface, it was evident that all cements containing the addition of up to 10 wt% Gyp had similar structures, although when 20 wt% Gyp was added to cement, large particles were present in the structure of the cement (figure 23) that looked similar to the shape and size of gypsum powder particles (see figure 15).

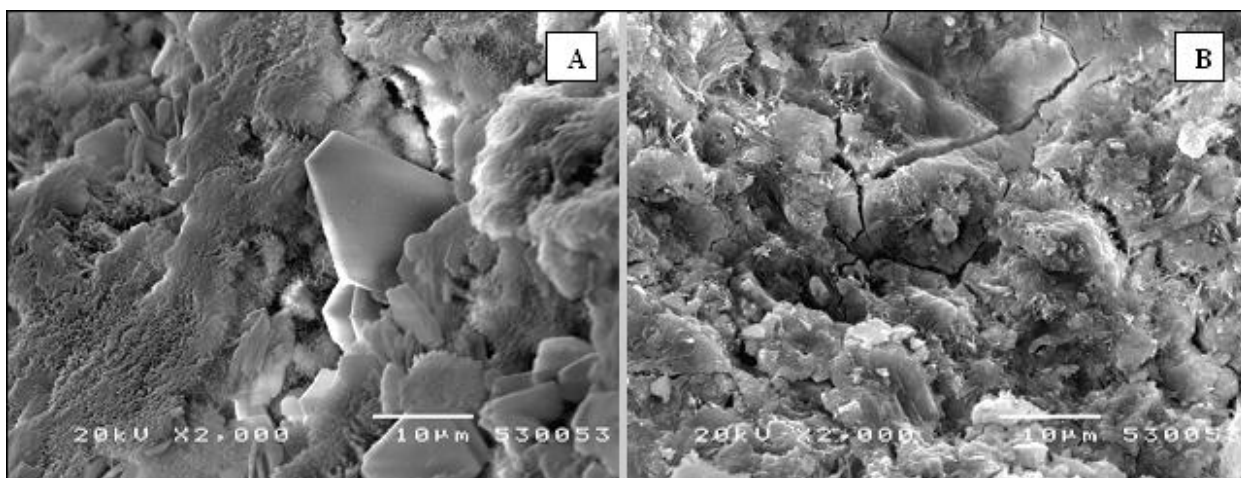


Figure 24: SEM photomicrographs of the fracture surfaces of the model cement (A) and cement containing 20 wt% CaSO_4 (B). More microcracks were evident in 20 wt% CaSO_4 -containing MC when compared with the control.

CaSO_4 -containing cements appeared to have a similar macrostructure as seen for the model cement although there was more porosity and the formation of more microcracks ($>20\ \mu\text{m}$ in length and $\sim 1\ \mu\text{m}$ in width) with increased CaSO_4 content to cement (figure 24).

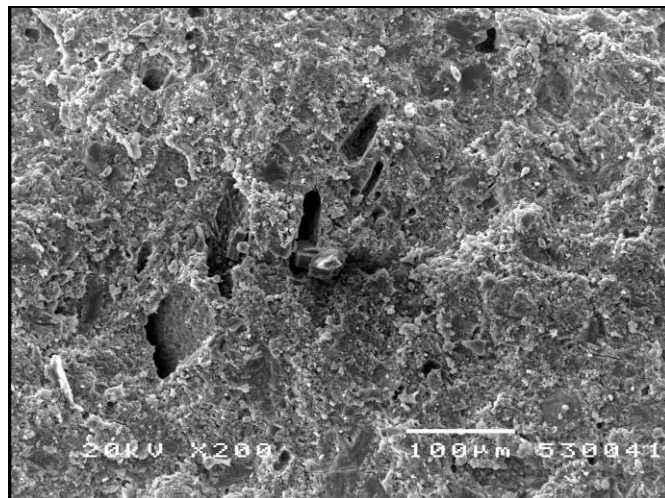


Figure 25: SEM photomicrograph of the fracture surface of 5 wt% PoP + 5 wt% CaSO_4 -containing cement, showing that large voids were present in the cement structure.

The structure of cements containing 5 wt% PoP + 5 wt% CaSO_4 (figure 25) showed large irregular-shaped voids ($<100\ \mu\text{m}$ in length, $\sim 20\ \mu\text{m}$ in width) in the structure which appeared to be randomly distributed.

4.3.3.2 *Outer surface of cement samples*

The microstructure of the outer surface (which would be the interface of the cement with tissues) of 5 and 20 wt% PoP-containing cements differed considerably and needle-like particles ($>10\ \mu\text{m}$) were more clearly visible in 5 wt% PoP containing cements than 20 wt% PoP-containing MC (figure 26). The crystal structure (x 5000 magnification) of the control cement appeared to consist of larger plates, with more voids and small needle shaped structures compared with the large needles in 5 wt% PoP-containing cements (figure 26).

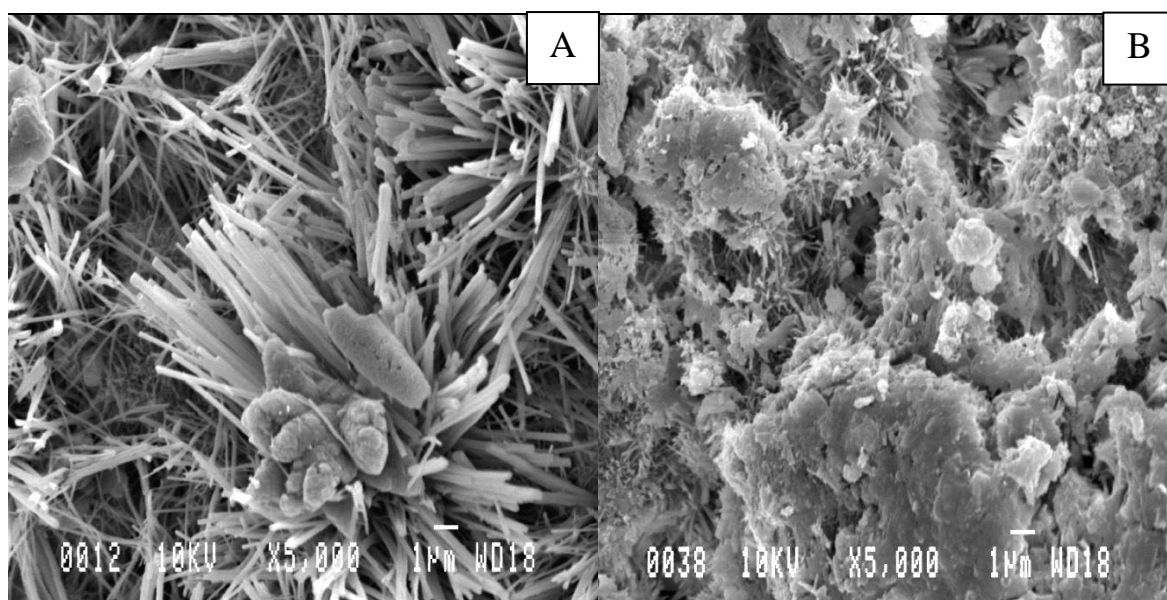


Figure 26: SEM photomicrographs of the outer surface of cement samples containing: A. 5 wt% PoP and B. 20 wt% PoP. Large needle like crystals were present in 5 wt% PoP-containing MC, however, none were visible in 20 wt% PoP-containing cements.

Clusters of needle-like structures were evident in cements containing 5 wt% and 10 wt% CaSO_4 , similar to those seen in cements containing 5 wt% and 10 wt% PoP. However, the needle-like structures were larger and more prominent in 5 wt% PoP-containing cements (see figure 26) than 5 wt% and 10 wt% CaSO_4 -containing MC.

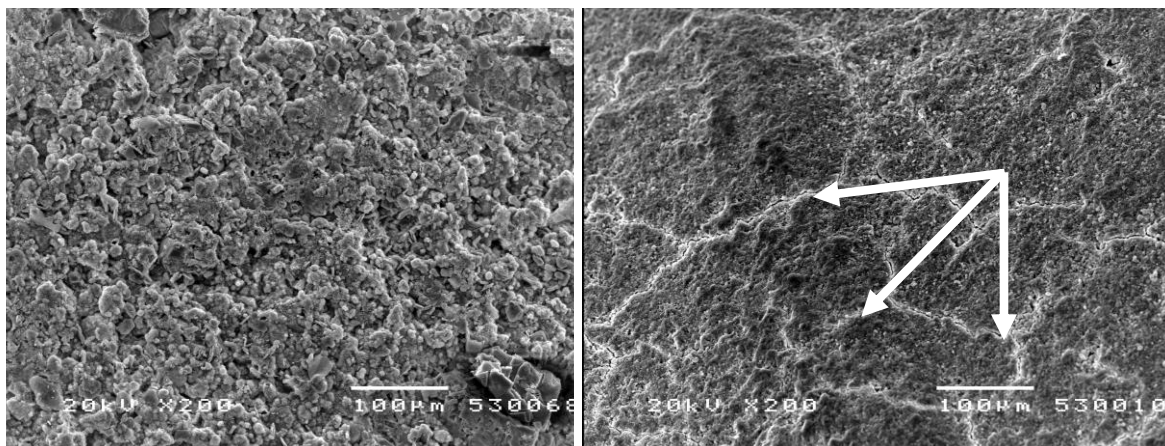


Figure 27: SEM photomicrographs of the outer surface of A: the model cement and B: 20 wt% CaSO_4 -containing cements. Cracks were present in cements containing 20 wt% CaSO_4 , which are indicated by the arrows.

Large microcracks were apparent in the structure of 20 wt% CaSO_4 -containing cement which were not seen in model cements without additions of calcium sulphates (figure 27).

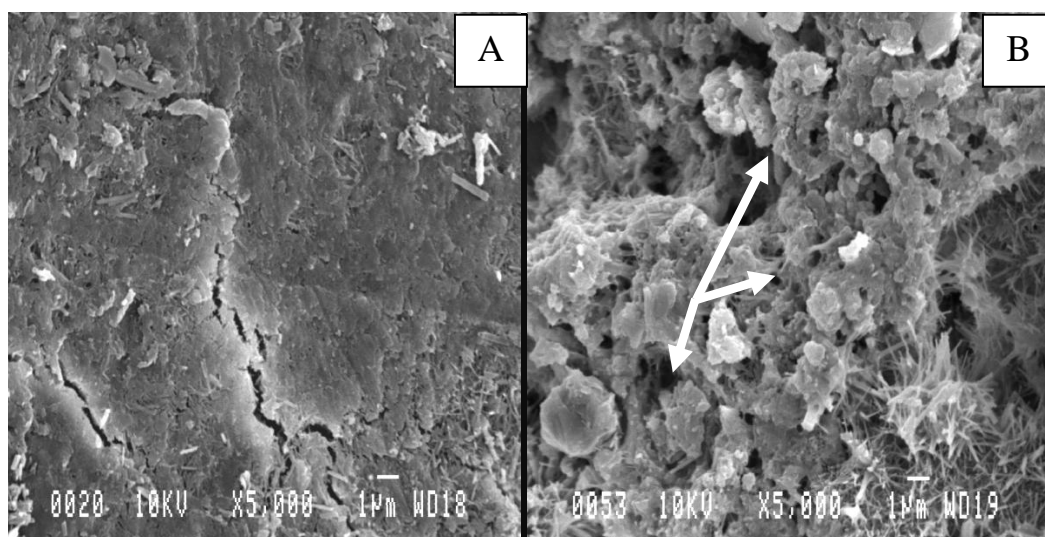


Figure 28: SEM photomicrographs of the outer surface of cement samples containing A: 5 wt% Gyp-containing cement and B: 20 wt% Gyp-containing cement. Pores (indicated by arrows) and a poorly-connected structure were seen in the SEM micrograph B of cements containing 20 wt% Gyp.

With increased addition of Gyp, more porosity and voids were evident, being similar to the effect of large additions of PoP and CaSO_4 . Some needle-like crystal clusters were present in samples

containing 20 wt% Gyp, which differed considerably in comparison with 5 wt% Gyp-containing cements (figure 28). PoP and CaSO_4 (Sigma) were selected for incorporation into model cements and MTA to carry out further studies.

4.4 *Addition of calcium sulphates to Snowcrete-based model cements*

4.4.1 **Effect on initial setting times**

The model for white MTA, SC containing Bi_2O_3 , and the SC model containing Gyp did not set within the investigated time period (>6 h). Additions of PoP decreased initial setting times of the white SC-containing model cement to only 38 ± 5 min with 5 wt% PoP additions and the setting times decreased further with increased PoP content to the model cement (see figure 29).

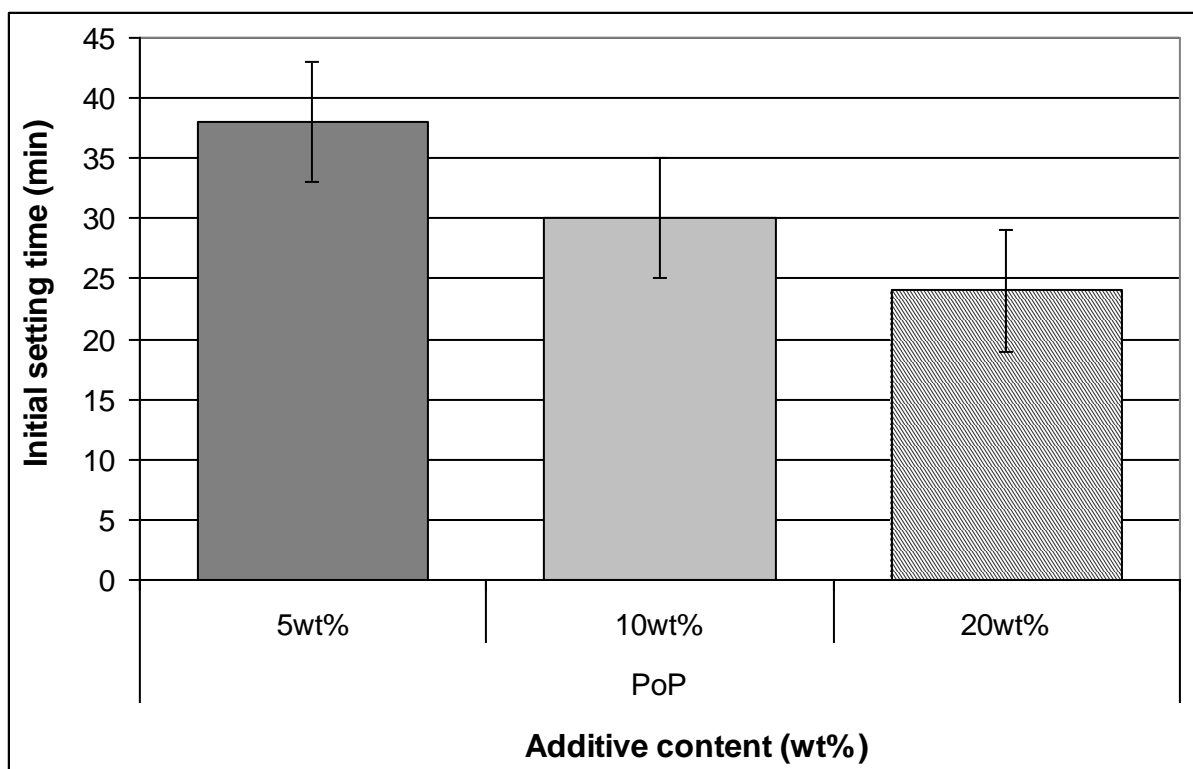


Figure 29: Initial setting times of the SC-model cement containing PoP. Cements without PoP and cements containing Gyp did not set in the investigated time period of 6 h. Error bars for setting time measurements were ± 5 min, which was the estimated error of the method.

4.4.2 Compressive strength and relative porosity

The white SC model or control for the experiment had a CS of 22.5 ± 6.5 mPa (see figure 30) and an RP of 23 ± 1 % (see figure 31) which were similar to the CS and RP of the MC model system, as shown in figures 20 and 21. Additions of PoP to white SC model cements caused the CS to increase considerably and CS remained higher than the model cement regardless of the quantity added (>20 wt%). RP remained lower than the model cement with PoP additions of less than 10 wt% to cement (figure 31) but increased with 20 wt% PoP additions.

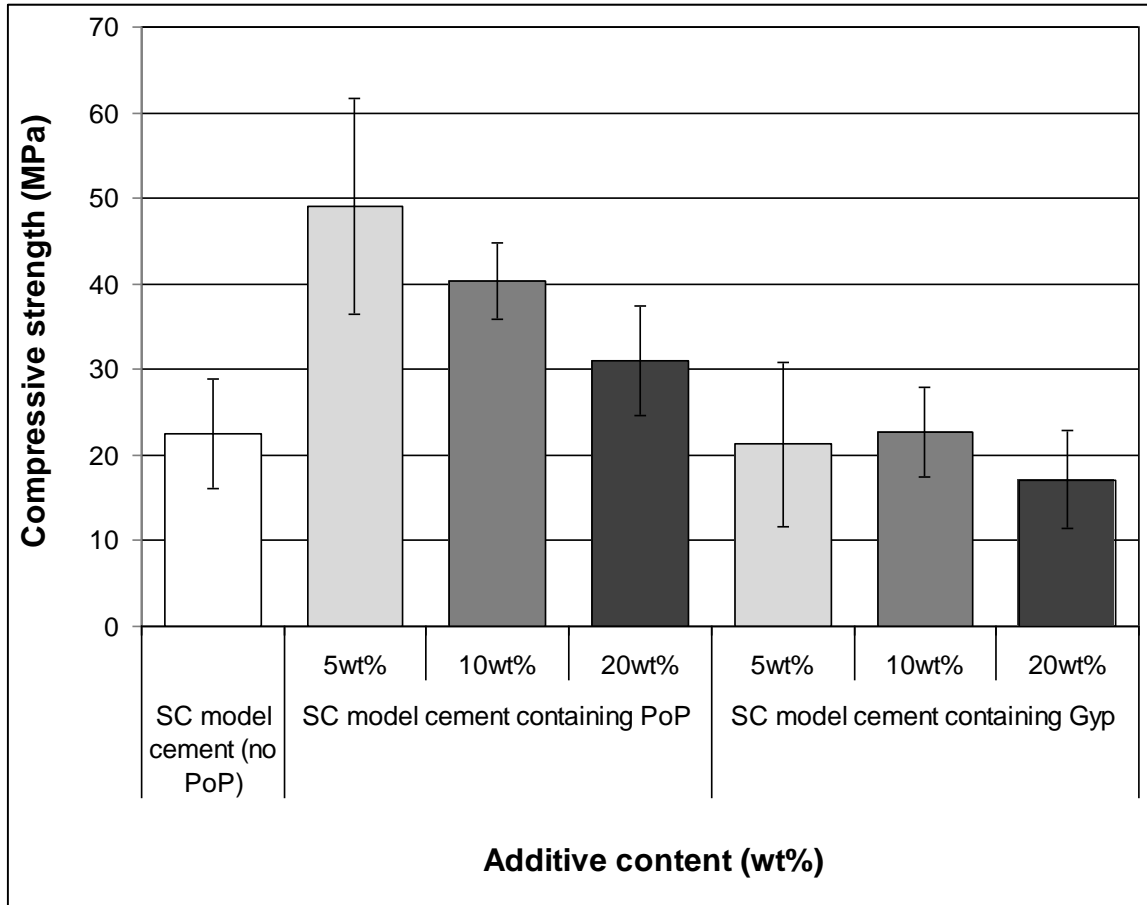


Figure 30: CS of the white SC-model cement with or without PoP additions. Cements with PoP had the highest CS compared with model cements and cements with Gyp additions. Standard deviations are present as error bars on the graph for the mean points plotted.

RESULTS

Gyp additions to white SC model cement had a similar CS to model cement with small additions (<20 wt%) but CS decreased to 17.1 ± 5.7 MPa with 20 wt% Gyp additions to white model cements (figure 30) and RP increased slightly (figure 31).

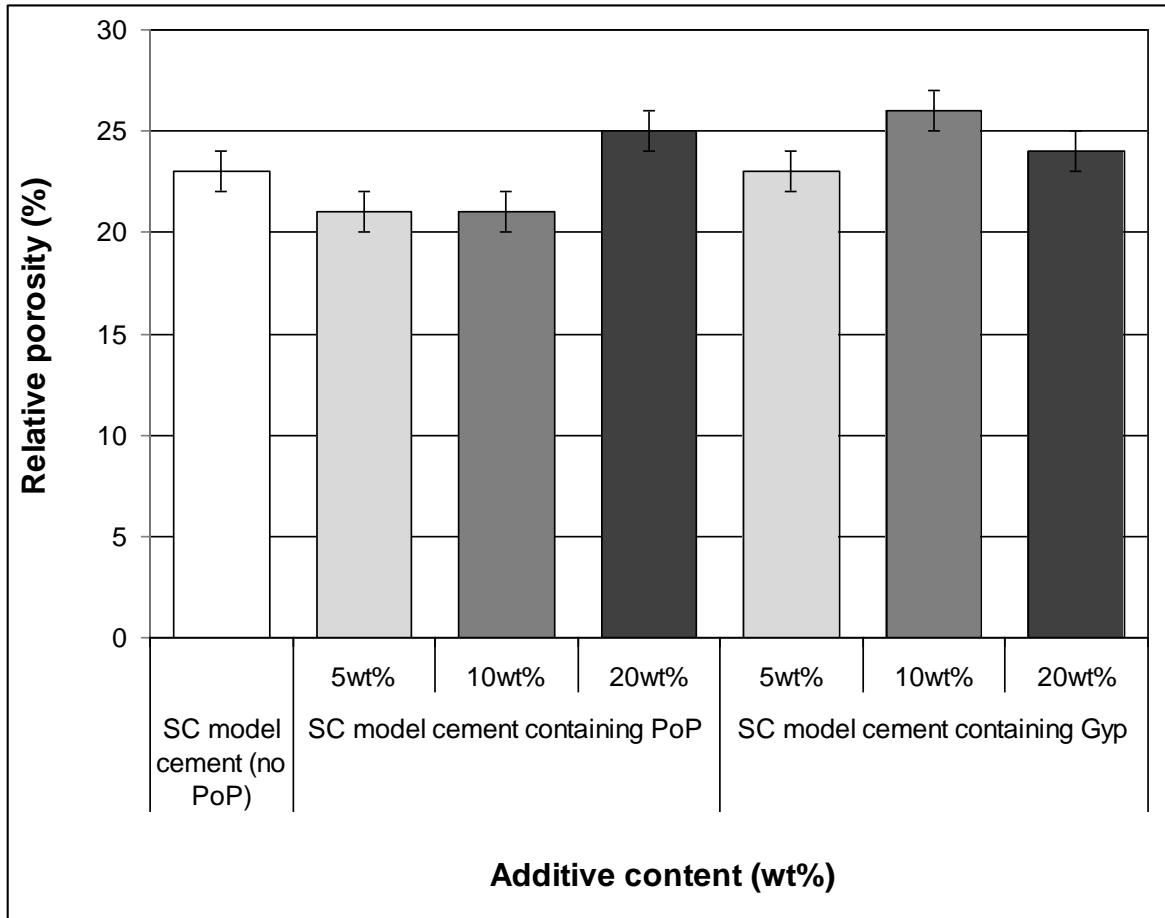


Figure 31: RP of the white SC-model cement containing PoP. Model cements with 5 and 10 wt% PoP additions had the lowest RP compared with the white SC-model cement with no additions and cements with Gyp additions. The approximate error of the method was ± 1 %.

4.5 *Plaster of Paris addition to mineral trioxide aggregate: effect on setting,*

CS and RP

4.5.1 Initial setting times of cements with PoP additions

The model cement and MTA did not set within the investigated time period (4 h) at room temperature. MTA containing PoP had similar initial setting times to the model cement (figure 32), 10 wt% additions of PoP reducing the setting time of MTA and the model cement to 40 ± 5 and 35 ± 5 min respectively.

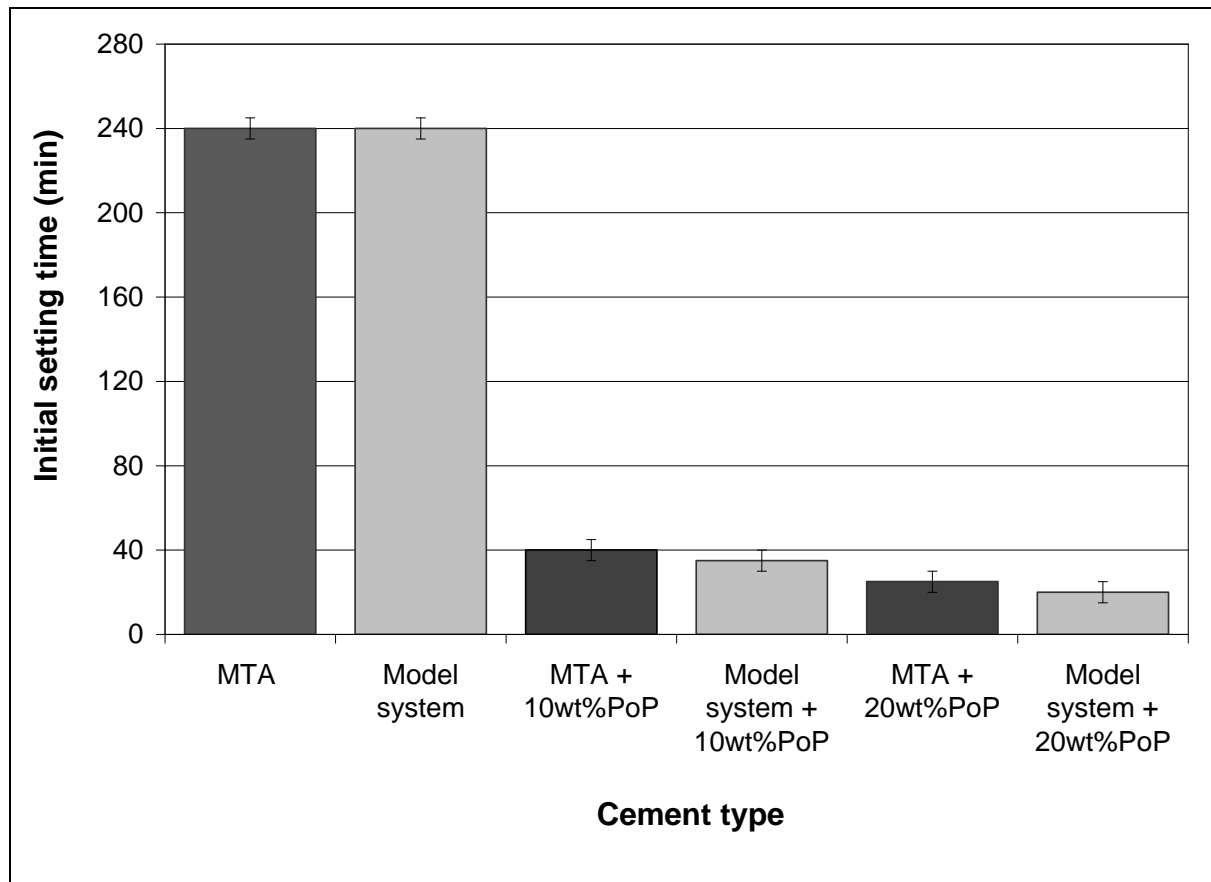


Figure 32: The effect of PoP additions on the initial setting time of the model system and MTA. Cements containing no addition of PoP set in over 4 h. MTA containing PoP was similar to the model cement containing PoP which was considerably reduced when compared with cement without PoP. The error bars represent the minimum and maximum error attached to the experimental method was ± 5 min.

Additions of 20 wt% PoP reduced the initial setting times of MTA and the model cement further to 25 ± 5 and 20 ± 5 min respectively.

4.5.2 CS and RP of MTA and the model system containing PoP

MTA had the highest CS of all cements and a RP that was much lower than that of the model cement for MTA (18 ± 1 %). MTA and the model system had similar CS although the CS of the model system was slightly higher than MTA but this was not statistically significant (figure 33).

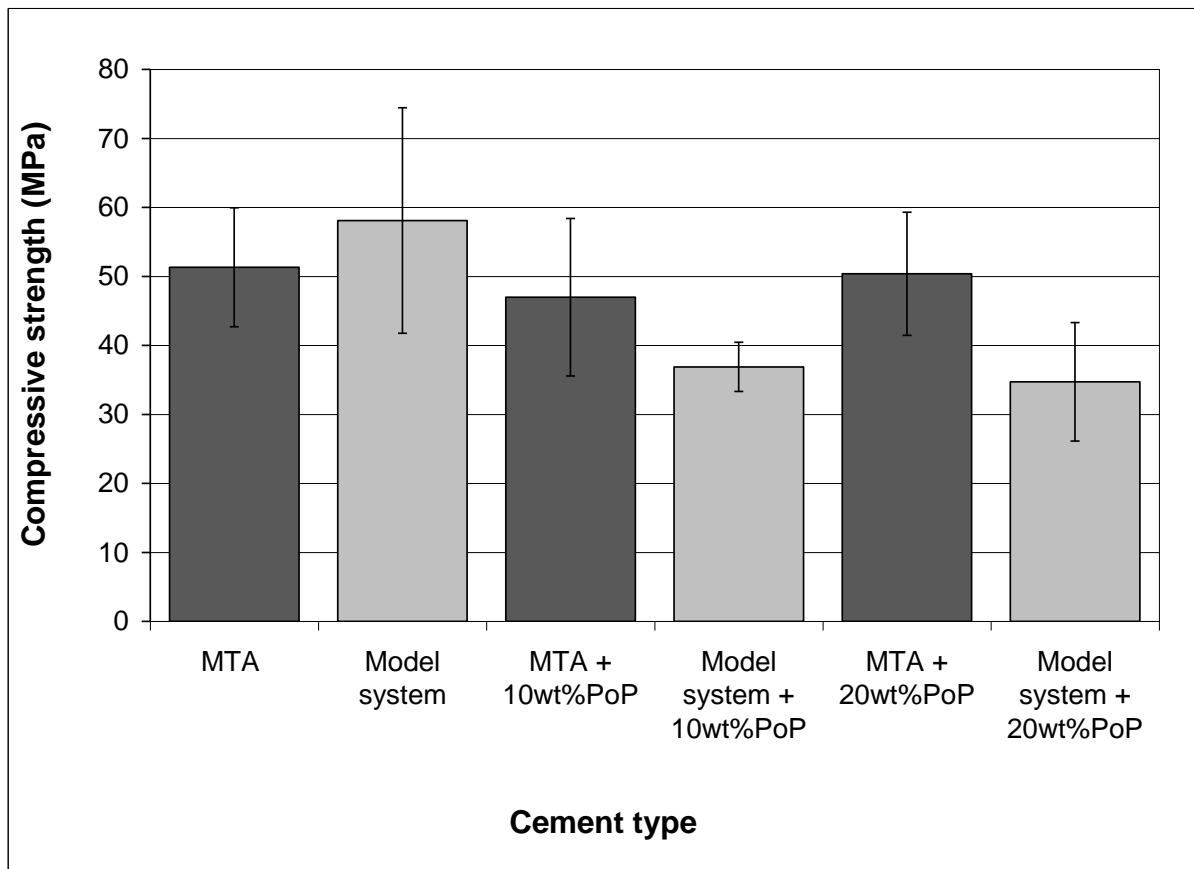


Figure 33: CS of MTA, the model system and cements modified with PoP. Similar CS was observed for MTA and the model system, however when cements were modified with PoP, the CS decreased the most for the model cement when compared with the CS of modified MTA. Standard deviations (\pm) were placed as error bars for each of the mean points plotted.

RESULTS

On addition of PoP, there was a considerable decrease in strength for the model cement, however, CS decreased only slightly for MTA, but was not significantly different ($p>0.05$), see figure 33.

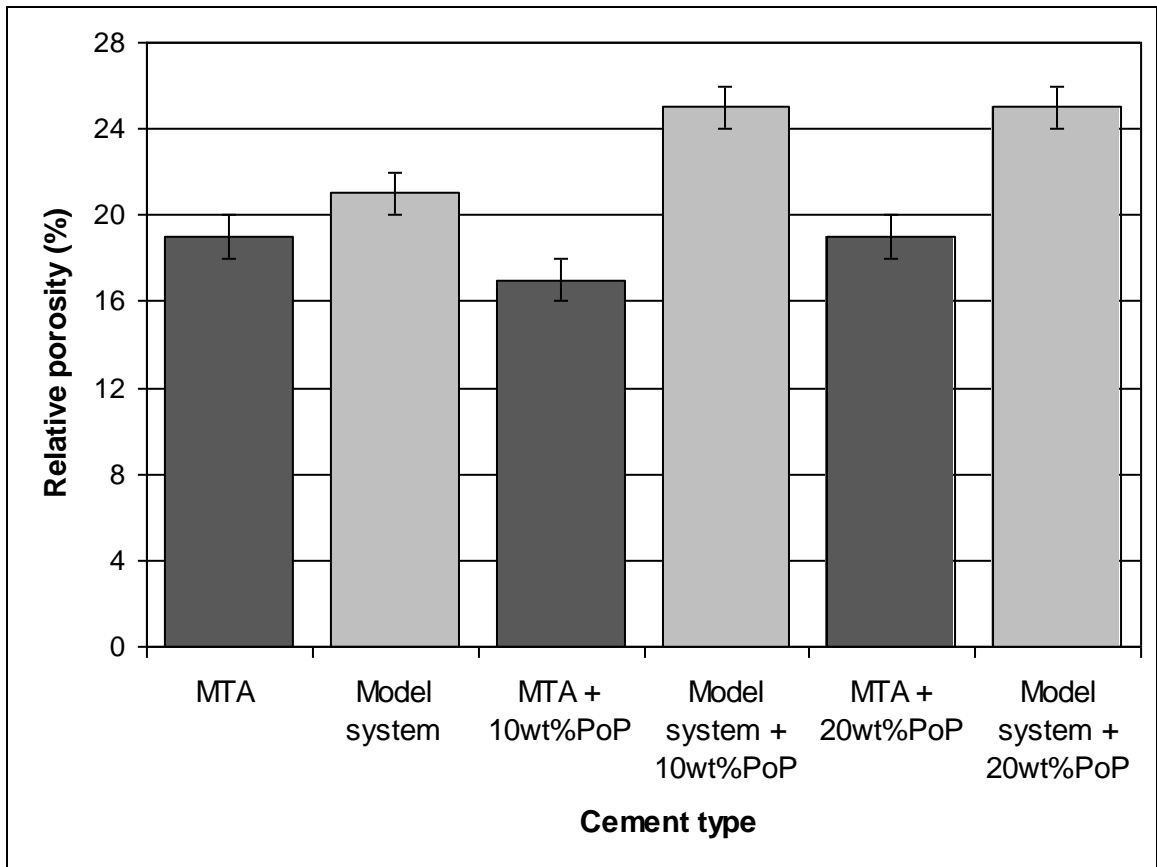


Figure 2: RP of MC and MTA cements containing additives of 10 wt% PoP. The addition of PoP increased the RP in cements. Error bars represent the total error of the method and calculations used to retrieve RP ($\pm 1\%$).

RP increased slightly with increased addition of PoP to model cements but remained similar for MTA even with the addition of PoP (see figure 34). The strut density of MTA was higher when compared with the strut density of model cement (see appendix 2). The strut density of unmodified MTA decreased with increasing PoP content, however, strut density increased for the model cement with the addition of 10 and 20 wt% PoP.

4.5.3 SEM examination of set structures

The outer surface of MTA revealed a more compact macrostructure, when compared with the outer surface of the unmodified model cement (figure 35).

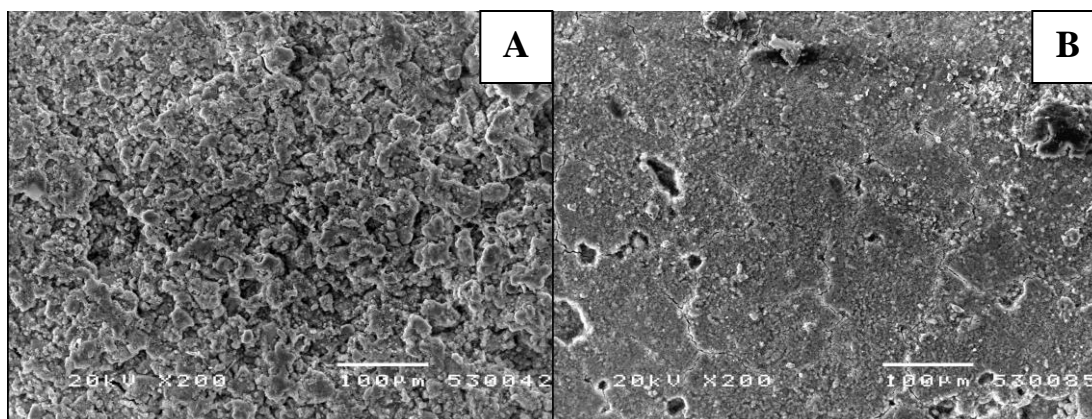


Figure 35: SEM micrographs of the outer surface of the model cement (A) and MTA (B) control samples, showing that MTA control had a more compact structure when compared with the model cement.

When the outer surfaces of MTA and the model cement were observed at x 2000 magnification, the differences between the two structures were apparent. MTA had small needle-like structures present on the surface, partially covering microcracks in the cement structure, whereas the model cement had large elongated particles present, forming a less compact structure (figure 35A) compared with the structure of MTA (figure 35B).

The outer surfaces of MTA and the model cement differed considerably when compared with the fracture surfaces (figure 36). The structure of the model cement had large crystal structures of $>10\text{ }\mu\text{m}$ in length, however, MTA was observed to have small needle-like structures ($<2\text{ }\mu\text{m}$ in length) on the surface of what appeared to be cement plates (figure 36, images A1 and B1). On the contrary, the fracture surface of the unmodified model cement appeared to be similar

to MTA, consisting of large cement plates (figure 36, images A2 and B2) apart from the presence of microcracks in the fracture surface of MTA. .

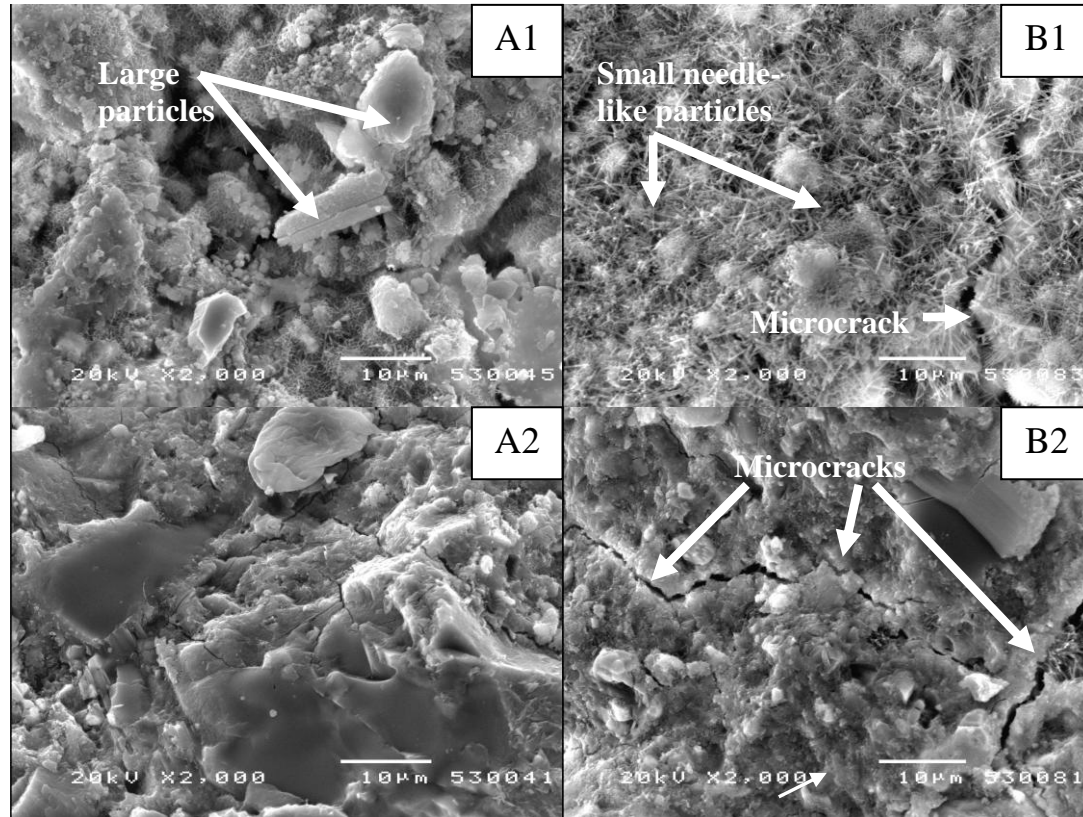


Figure 36: SEM photomicrographs comparing the differences between the outer surface (1) and fracture surfaces (2) of the model cement (A) and MTA control (B) samples. MTA had small, needle-like particles present on the outer surface (B1) which differed to the model cement which had mainly large particles on the outer surface (A1). Microcracks were seen in the SEM photomicrograph of the outer (B1) and fracture surfaces (B2) of MTA.

The addition of 10 wt% PoP altered the microstructure of the model cement and MTA considerably (figure 37, images A1 and B1) when observing the outer surface of these cements, since there appeared to be large crystals present upon cement plates covered with small needle-like structures which contrasted with the images from the outer surfaces of unmodified cements (figure 36, images A1 and B1). Little difference was seen when comparing the outer structures of the model cement and MTA containing 10 wt% PoP (figure 37).

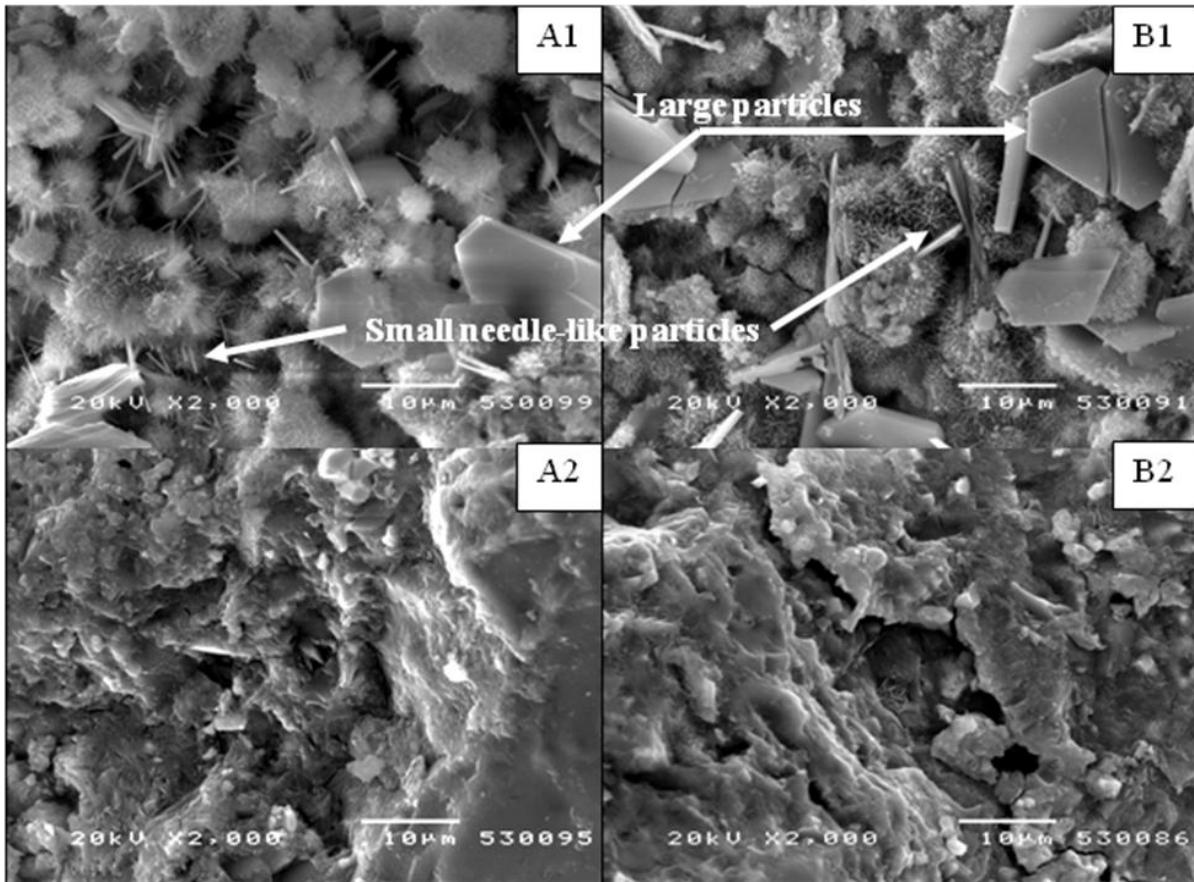


Figure 37: SEM photomicrographs showing the effect of 10 wt% PoP addition on the outer (1) and fracture surfaces (2) of the model cement (A) and MTA (B). The white arrows in images A2 and B2 point to the large crystals present within modified model cement and modified MTA. A similar fracture surface was apparent for model cement and MTA that both contained 10 wt% PoP (A2 and B2) which contrasted the appearance of the outer surfaces of these cements that contained 10 wt% PoP (A1 and B1).

Cements modified with 10 wt% PoP had similar fracture surfaces (figure 37, images A2 and B2) when compared with cements that were unmodified (figure 38, A2 and B2) and there was no apparent difference in the appearance of the fracture surfaces of the model cement and MTA containing 10 wt% PoP.

4.6 Analysis of the crystal structures of the modified model system and mineral trioxide aggregate using XRD

The main crystalline phases found from XRD analysis of the model cement modified and unmodified with calcium sulphates were Bi_2O_3 , ettringite, calcium hydroxide and traces of gypsum, the latter only in samples with high calcium sulphate additions (figure 38). All cements combined the same amount of Bi_2O_3 (20 wt%), therefore the Bi_2O_3 -peak was used for calibration of the patterns.

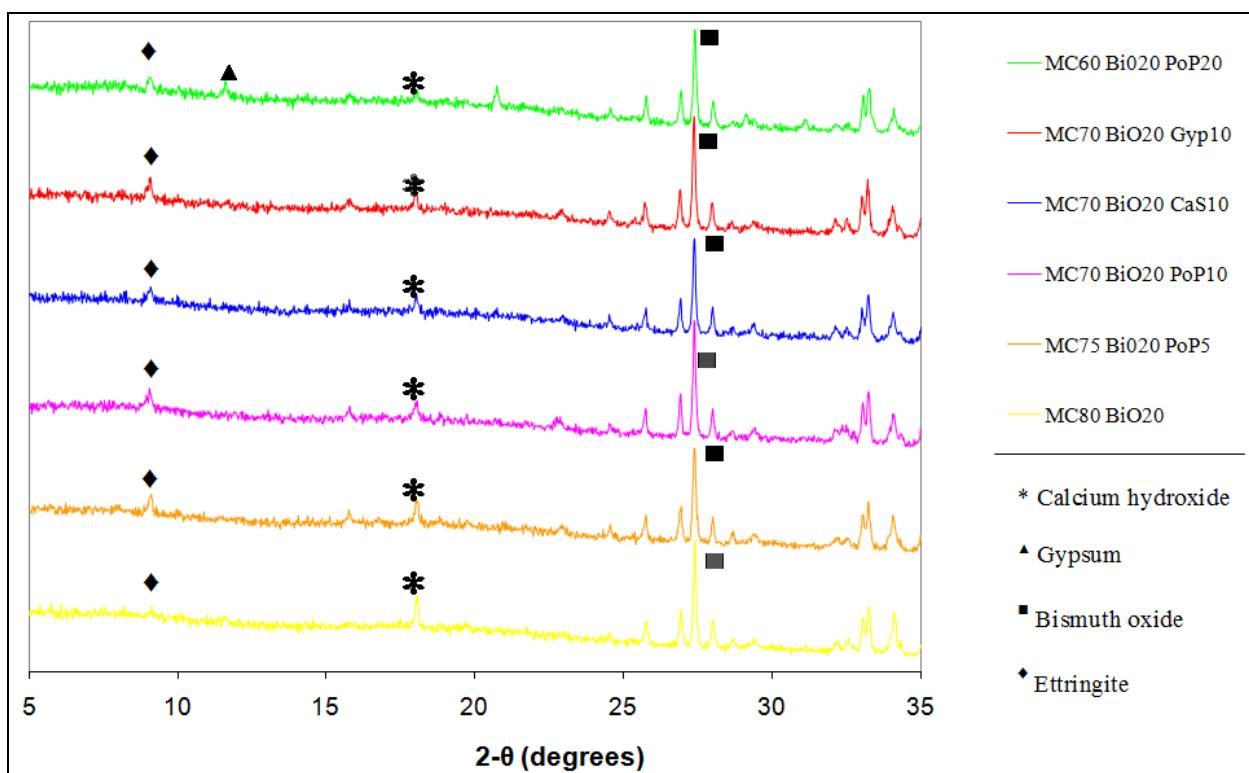


Figure 38: XRD patterns of cements containing various amounts of calcium sulphates. Residual gypsum was detected in 20 wt% PoP-containing cements. All cements showed some degree of ettringite formation and the presence of calcium hydroxide.

Observing the XRD peaks from patterns, there was a relatively smaller ettringite peak in the crystalline structure of control model cements when compared with cements containing calcium sulphates (figure 38). Cements with 5 and 10 wt% PoP, 10 wt% CaSO_4 and Gyp

additions appeared to have relatively similar sized peaks from the XRD patterns observed, which corresponded with the presence of ettringite, which may have indicated that relatively similar amounts of ettringite crystals were present in crystalline structures.

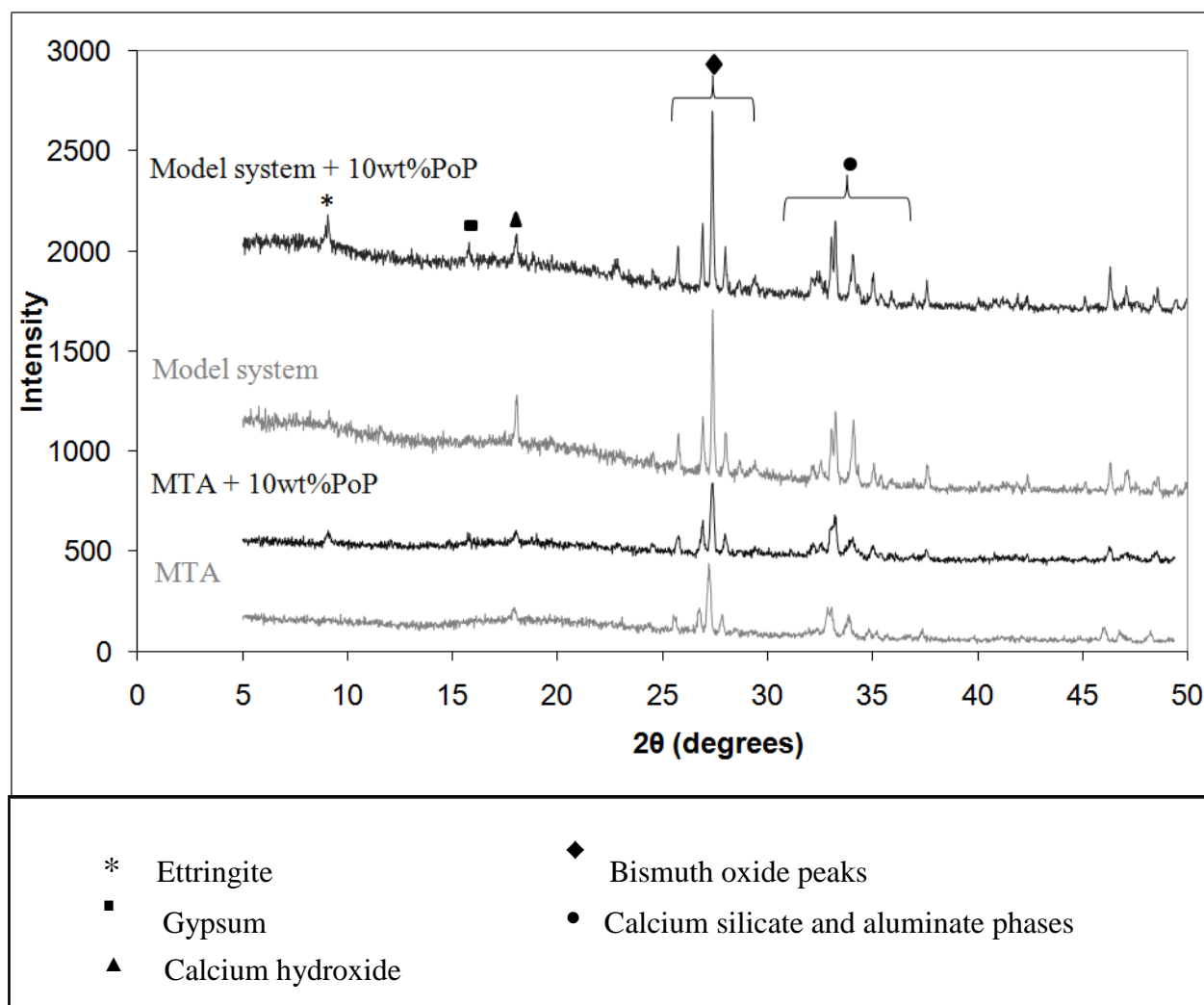


Figure 39: X-ray diffraction patterns for MTA, the model system and cements containing 10 wt% PoP. MTA and the model system had the same crystalline phases present, both showing additional gypsum and ettringite phases in the cements with PoP addition.

Cements containing 20 wt% PoP had a relatively smaller size ettringite peak compared with other additive-containing cements but showed a stronger ettringite peak present compared with the control. Gypsum was only detected in cements containing 20 wt% PoP and there was only a

RESULTS

small peak for calcium hydroxide evident in the XRD patterns in figure 39. In 10 wt% PoP-, CaSO_4 - and Gyp-containing cements, smaller peaks for calcium hydroxide prevalence in the crystalline structures were detected compared with calcium hydroxide peaks for 5 wt% PoP-containing cement and the control, which had the highest calcium hydroxide peaks.

The same main crystalline phases (calcium hydroxide, silicate and aluminates) were present in MTA and the model system (figure 39) and Bi_2O_3 was detected in both indicating the similar chemical composition of the two cements. When 10 wt% PoP was added to MTA and the model system, the same calcium and aluminate phases were present, calcium hydroxide and bismuth oxide were also present, as seen in figure 39. Ettringite and gypsum were detected in the modified model cements and modified MTA, which were not detected in unmodified model cements and unmodified MTA (figure 39).

For the remainder of the study, the effect of 10wt% PoP additions was selected for intense subsequent studies and to observe the extent of the effect of PoP, 30wt% PoP additions were also incorporated into model cements and MTA for comparison with 10wt% additions.

4.7 Kinetic solubility of calcium sulphate additives

The concentration of Ca^{2+} ions increased rapidly within the solution during the first 5 min of calcium sulphate immersion in water (figure 40), from 0 to >0.008 M of Ca^{2+} ions (figure 35).

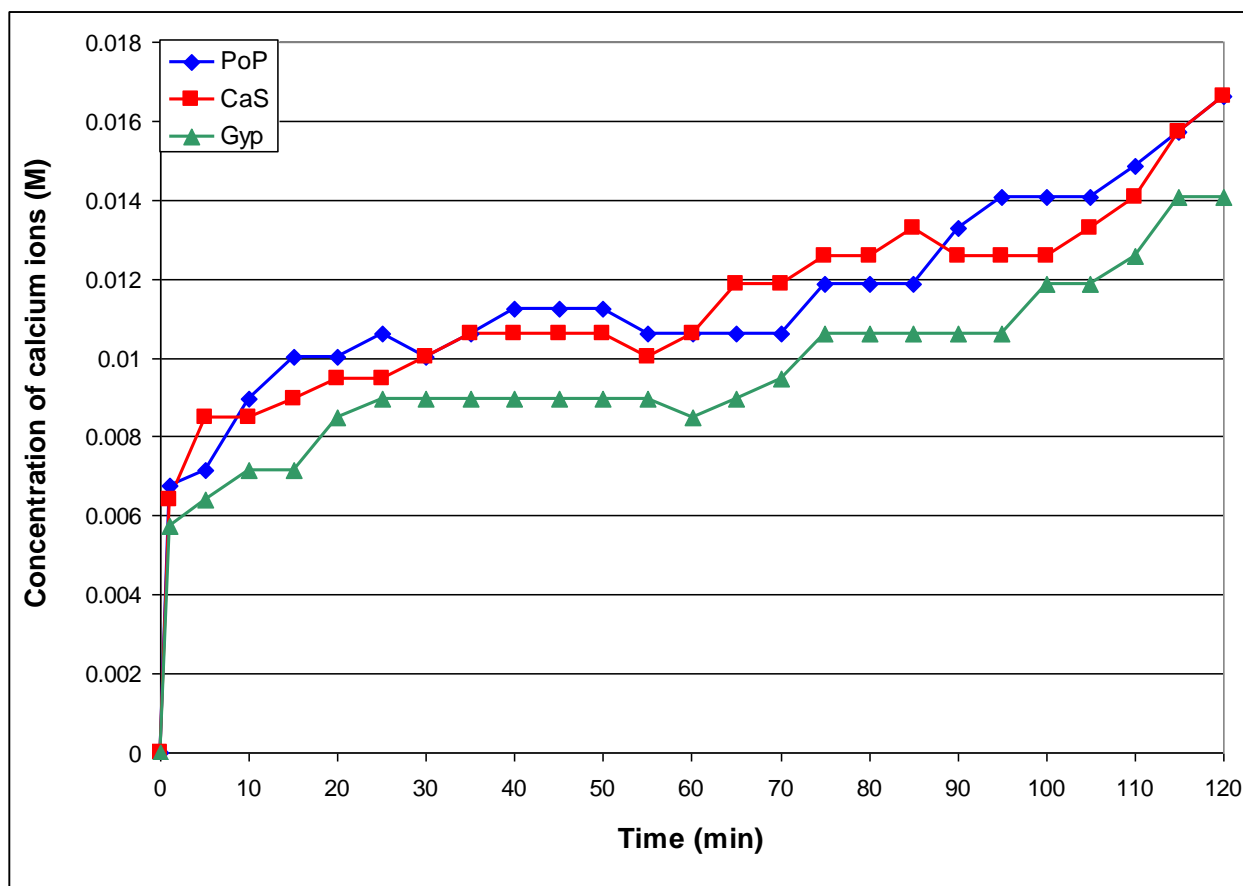


Figure 40: Graph showing the molar concentration of calcium in water (from calcium sulphates) with time. PoP had a similar kinetic solubility to CaSO_4 which was more soluble than Gyp. Approximate error of the method was $\pm 20\%$.

After 15 min, the concentration of Ca^{2+} ions in PoP-containing solution was slightly higher than that of the CaSO_4 -containing solution, which in turn had a higher concentration of Ca^{2+} than Gyp-containing solution. PoP and CaSO_4 showed a higher rate of dissolution than Gyp throughout the experimental time period (figure 40).

5 Preliminary work: Sterilisation of powders/ set cements

Model cements placed into the autoclave wet or dry had significantly lower ($p < 0.05$) CS and increased RP when compared with the CS and RP of the model cement that had not been autoclaved.

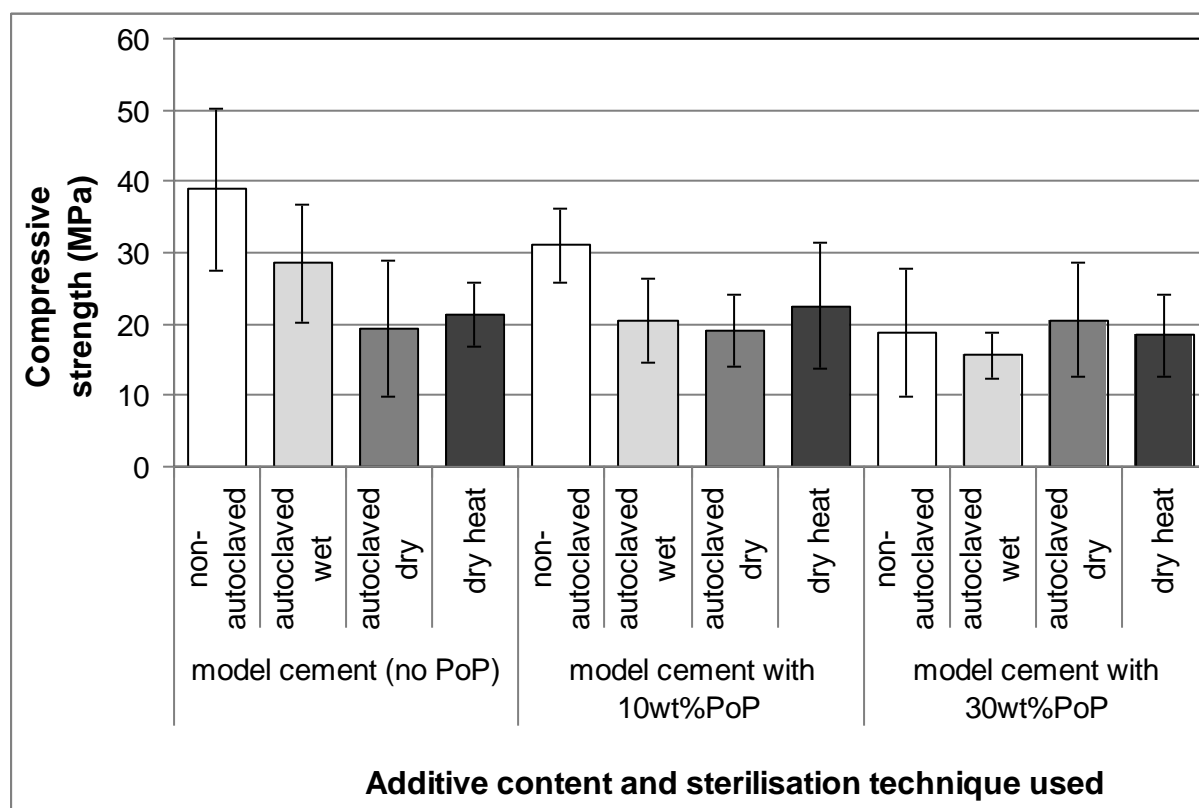


Figure 41: CS of model cements with or without PoP additions that were sterilised using different treatments. CS generally decreased when cements were sterilised when compared with cements that were not sterilised. Standard deviations of mean values are present on the graph as error bars.

Cements that were sterilised via dry heat treatment of powders had a significantly lower CS ($p < 0.05$) when compared with the model cement that had not been sterilised (see figure 41) and an RP similar to the non-sterile model cement (figure 42). CS decreased significantly for cements containing 10 wt% PoP that were wet or dry autoclaved ($p < 0.05$) and RP also increased considerably for 10 wt% PoP-containing cements samples which were autoclaved compared with

RESULTS

cements that had not been sterilised that contained 10 wt% PoP (figure 41). On the contrary, cement samples containing 30 wt% PoP that were sterilised using dry heat or autoclaved either wet or dry had similar CS and RP to 30 wt% PoP-containing cements that were not sterilised (figures 41 and 42).

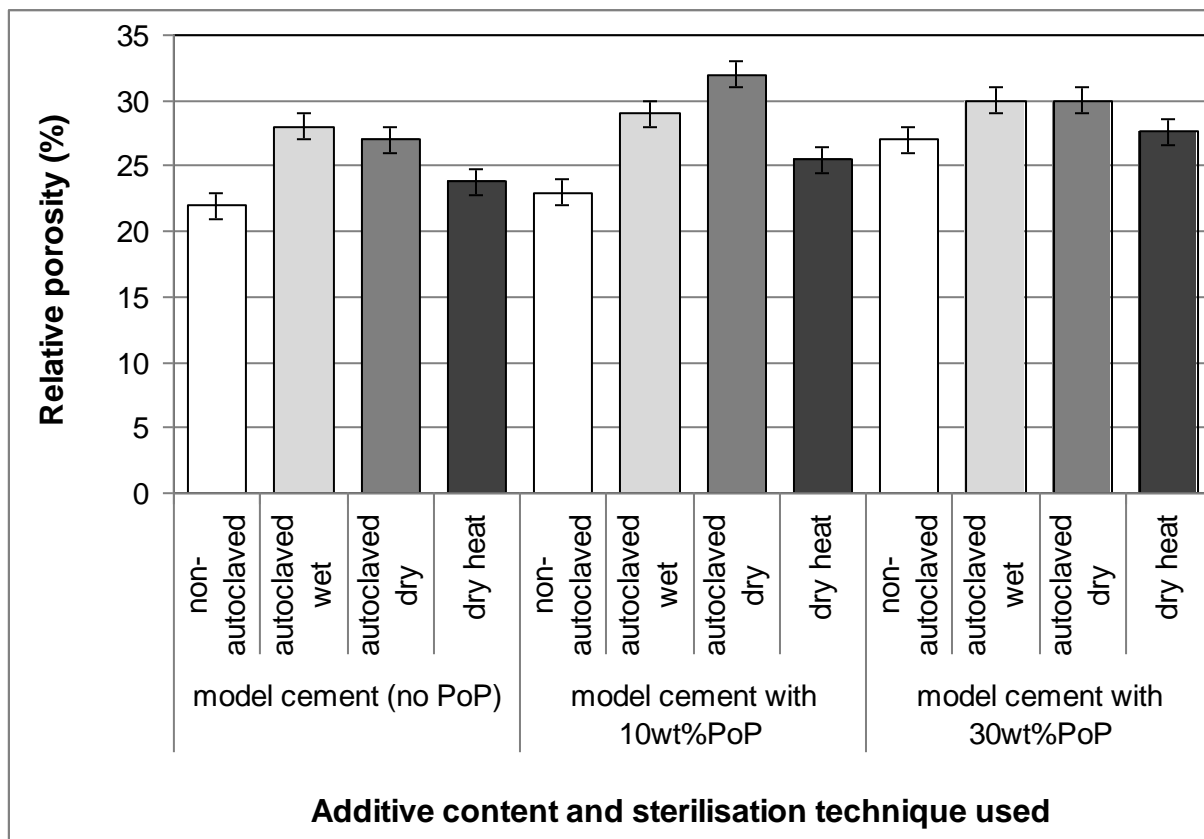


Figure 42: Bar chart to represent RP of cements with or without the addition of PoP which were sterilised using different methods. Error bars represent the approximate error of the method and calculations used to retrieve RP ($\pm 1\%$). RP were higher for cements that had been autoclaved or had treated with dry heat than those which were not sterilised.

Following the results of these studies, cement powders were sterilised via gamma irradiation for successive studies rather than the use of the other methods explored in this section for sterilising cement powders and samples.

6 Effect of ageing on the stability and solubility of modified cements

6.1 *Long term stability of cements modified with Plaster of Paris*

6.1.1 **Effect of water storage on CS and RP of modified grey model cements**

At 10 and 30 days, CS remained similar for MC model cements with no PoP and cements containing 10 or 30 wt% additions of PoP (figure 43). RP of 30 wt% PoP-containing cements stored for 10 days in water were higher than cements containing no PoP and containing 10 wt% PoP (figure 44) but RP decreased for all cements when obtained at 30 days compared with 10 days. Model cements set for 30 days in water had the lowest RP when compared with PoP-modified cements which were also set for 30 days (figure 44).

CS significantly increased ($p < 0.05$) for the model cement and cements containing 10 wt% PoP additions after 3 months of storage, compared with the low CS of 30 wt% PoP when set for 3 months and remained higher than the CS of 30 wt% PoP-containing cements throughout the experiment. CS of 30 wt% PoP-containing cements significantly decreased ($p < 0.05$), from 28.6 ± 5.7 MPa at 3 months to 0.9 ± 0.3 MPa after 12 months of storage in water (figure 43), which was significantly lower than CS of model cements and 10 wt% PoP-modified cements ($p < 0.05$). (figure 44).

RP decreased for 10 wt% PoP-containing cements from 21 ± 1 % when stored for 30 days to 17 ± 1 % after 3 months of storage in water (figure 44) which was unlike model cements and 30 wt% PoP-modified cement. RP of 10 wt% PoP-containing cements remained similar to the unmodified grey model cement unlike the RP of 30 wt% PoP additions which increased with water storage time (figure 43).

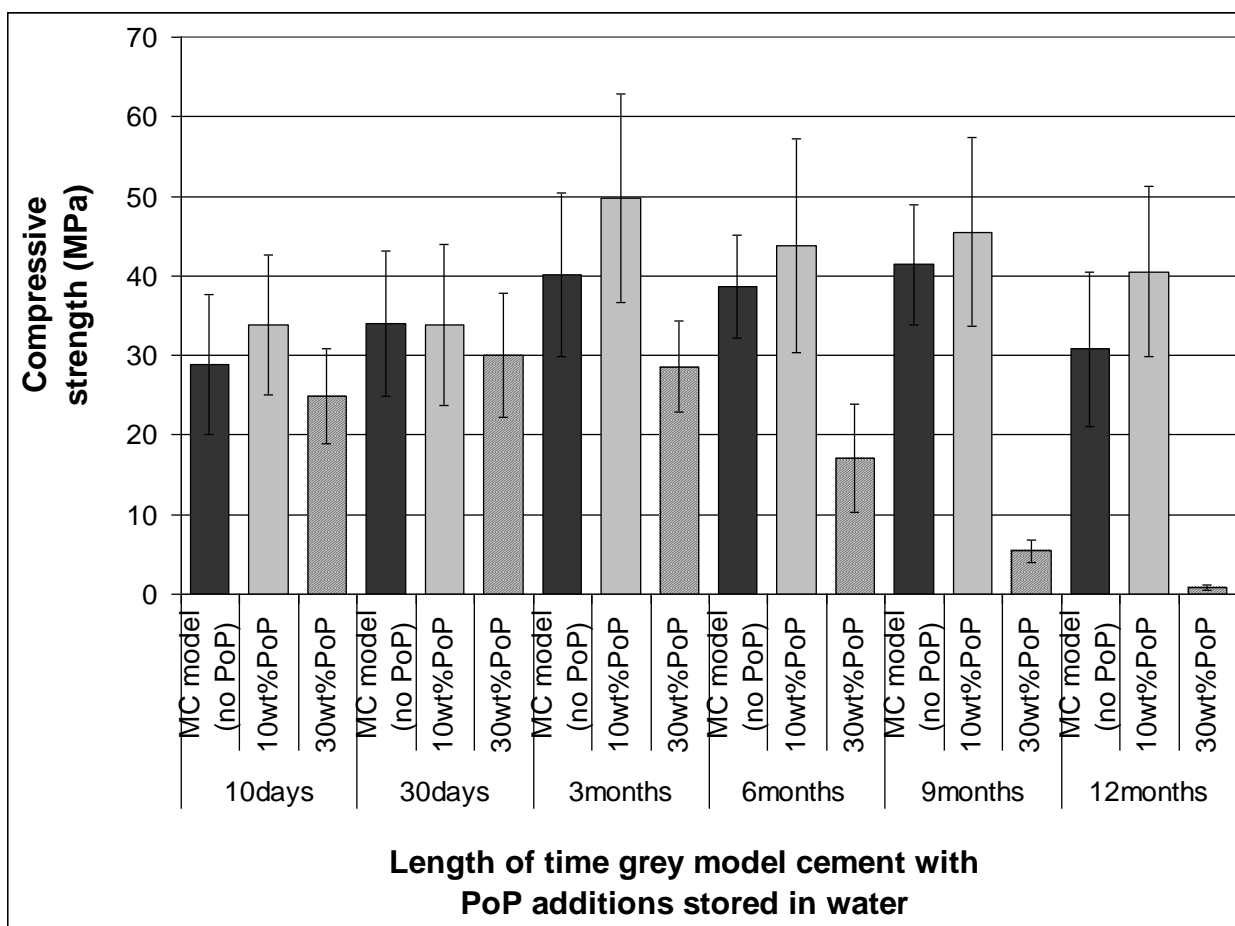


Figure 43: CS of grey model system with or without additions of PoP, stored in distilled water for up to 12 months. Error bars on the graph represent the standard deviations for mean values that were plotted. Strength declined considerably with length of storage time for 30 wt% PoP-containing cements.

Dry densities were observed to be lower for cements with added PoP. After 10 days of setting, the dry densities for cements containing 10 and 30 wt% PoP were lower than cements without PoP. When storage time for cements without PoP addition increased from 10 days to 3 months, the dry density increased. However, after 3 months of storage up until the end of the experimental period of 12 months, the dry density of model cements decreased. Similarly, the dry density increased for model cements containing 10 wt% additions of PoP up to 3 months of storage but then decreased notably after this time. On the contrary, model cements with 30 wt% PoP additions had decreased dry densities with increased storage time (see appendix 3.1).

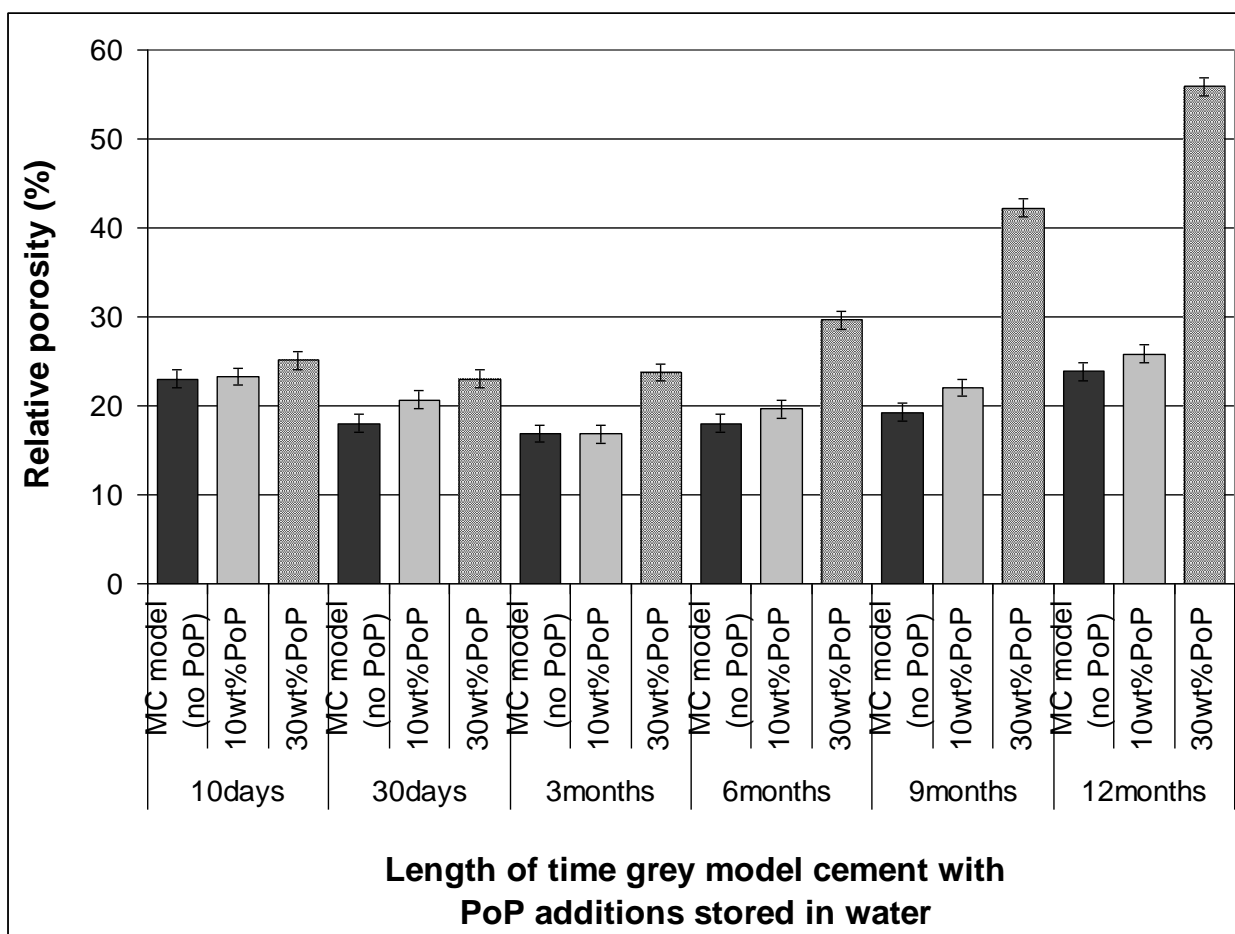


Figure 44: RP of grey model cements with or without added PoP which were stored in distilled water for up to 12 months. Error bars represent the approximate error of the method and calculations used to retrieve RP ($\pm 1\%$). RP were observed to increase noticeably when the model cements containing 30 wt% PoP were stored with time.

6.1.2 Effect of water storage on CS and RP of modified white model cements

CS and RP were similar for unmodified and 10 wt% PoP-containing white cement stored for up to 9 months and the CS and RP of 10 wt% PoP-containing cements showed to be slightly higher than the CS of model cements throughout the experiment (see figure 45 and 46). When the additive content for the model cement increased to 30 wt% PoP, there was a decrease in CS and increased RP compared with the model cement of which were more profound with increased storage time.

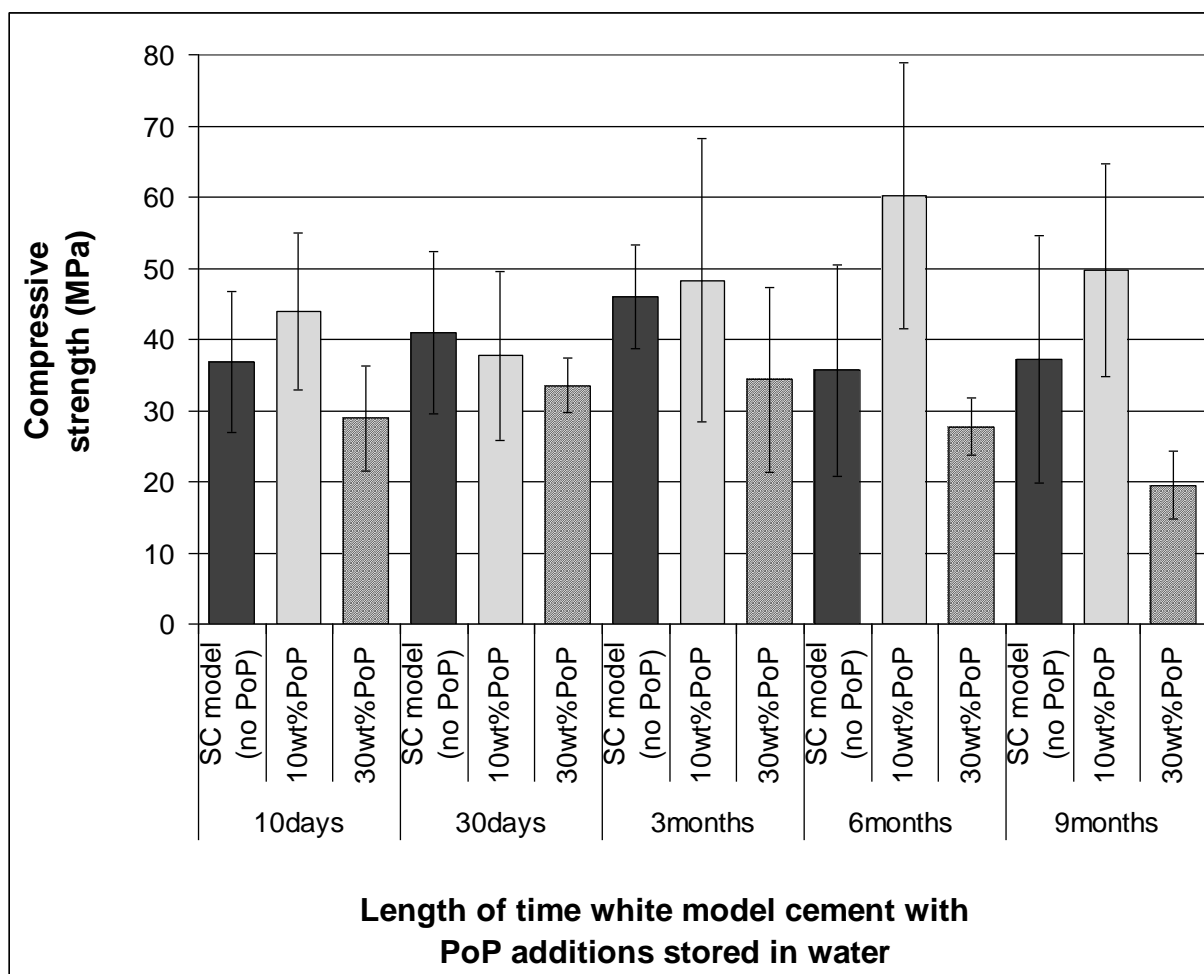


Figure 45: CS of white model cement with or without PoP additions at various time points. Error bars on the graph represent the standard deviations for mean values that were plotted. 10 wt% PoP additions to the white model cement increased slightly with increased storage time in distilled water.

The dry densities of the white model cement without PoP additions were generally higher than cements with PoP additions and no clear trend was observed for unmodified cements stored in water with time. Dry density decreased when PoP was added to the white model system, at 10 and 30 wt% and generally decreased with time (see appendix 3.2).

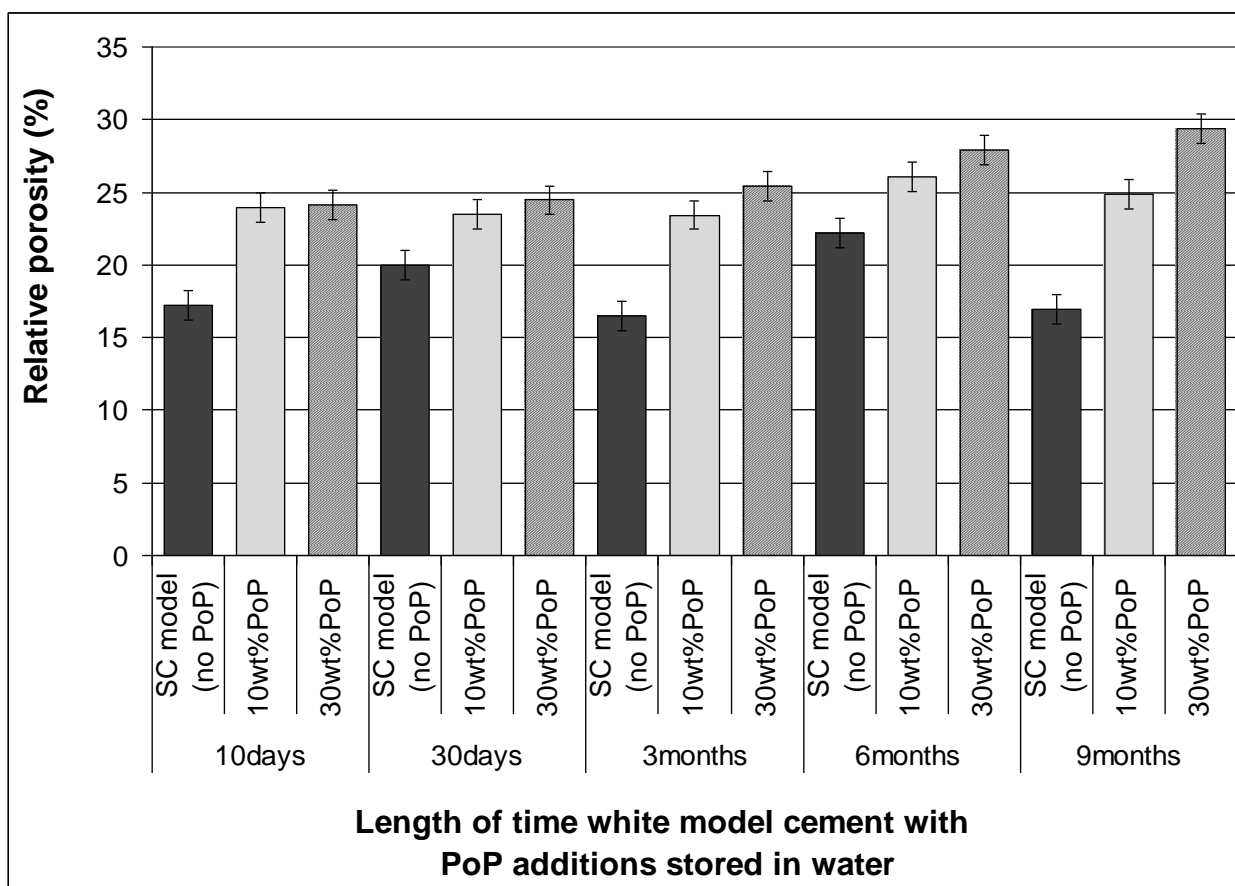


Figure 46: RP of the white model cement and cements containing PoP additions stored in distilled water. Error bars represent the approximate error of the method and calculations used to retrieve RP ($\pm 1\%$). Cements with PoP additions tended to have higher RP when compared with the white model cement only. Cements with PoP additions had increased RP with increased storage time.

6.1.3 Effect of storage in medium on CS and RP of modified grey model cements

Storage of unmodified cements and 10 wt% PoP-modified cements in culture medium had similar CS throughout the 9 month study (figure 47) compared with CS of modified or unmodified grey model cements stored in distilled water (figure 43). The exception to the CS trend for culture medium storage was that after 6 months of storage, model cements containing 10 wt% PoP had a significantly higher CS compared with model cement stored in culture medium ($p < 0.05$). The grey model cement had a similar CS when stored in culture medium

RESULTS

compared with the CS of grey model cement stored in distilled water for 10 days and had the same RP (figure 48). RP of model cements and 10 wt% PoP-containing cements decreased with increased storage time in culture medium but the opposite trend was identified for 10 wt% PoP-modified or unmodified model cements since RP increased slightly with increased storage time in distilled water (figure 44).

CS of 30 wt% PoP-modified model cements remained similar to the model cement throughout the 9 month study of cements stored in culture medium, but CS was significantly lower ($p < 0.05$) for 30 wt% PoP containing cements than model cements when stored in culture medium for 9 months (figure 47). RP increased considerably with increased storage time in distilled water for 30 wt% PoP-modified cements (figure 44) but RP remained the same for 9 month-culture medium stored cement with 30 wt% PoP as the RP of 30 wt% PoP-modified cement after 30 days of culture medium storage (figure 48). Cements with 30 wt% PoP always had a slightly higher RP throughout the 9 month study for samples stored in culture medium compared with model cement and 10 wt% PoP containing cements that were stored in culture medium also.

30 wt% PoP-containing cement that had been stored in culture medium for 9 months had significantly higher CS ($p < 0.05$) than the CS of model cements containing 30 wt% PoP stored for the same length of time in distilled water (see figure 43). However, RP increased considerably to when model cements with 30 wt% PoP stored in distilled water for 9 months (see figure 44) but remained low for model cements containing 30 wt% PoP that had been stored in culture medium for 9 months (figure 47).

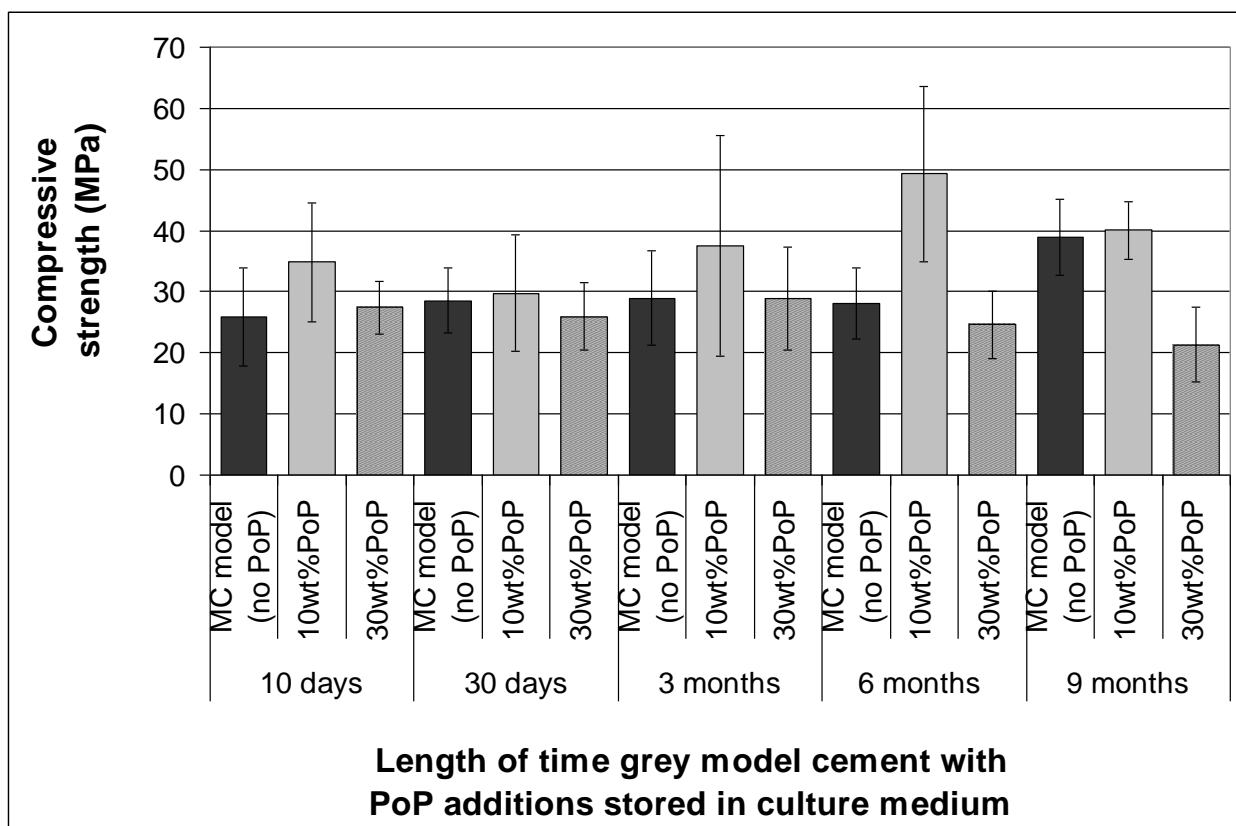


Figure 47: Bar char to represent CS of the grey model cement with or without PoP and set for different lengths of time in culture medium. Error bars on the graph represent the standard deviations for mean values that were plotted. Cements with or without 30 wt% PoP additions had similar CS at 10, 30 days and 3 months. However, the highest CS were observed for cements containing 10 wt% PoP additions.

Dry densities of model cements stored in culture medium were the highest compared with the densities of model cements containing PoP stored in distilled water (see appendix 3.1). Dry densities decreased slightly for unmodified PoP cements stored for 30 days compared with 10 days. However, the dry density increased slightly after 30 days when the model cement contained 30 wt% PoP (see appendix 3.1) whilst no change was observed for 10 wt% PoP-containing cements stored for 30 days when compared with 10 days. Cements containing 10 wt% PoP and the model cement without PoP had dry densities that increased slightly after 3 months of setting but remained similar for cements with 30 wt% PoP but lower than that of unmodified cement.

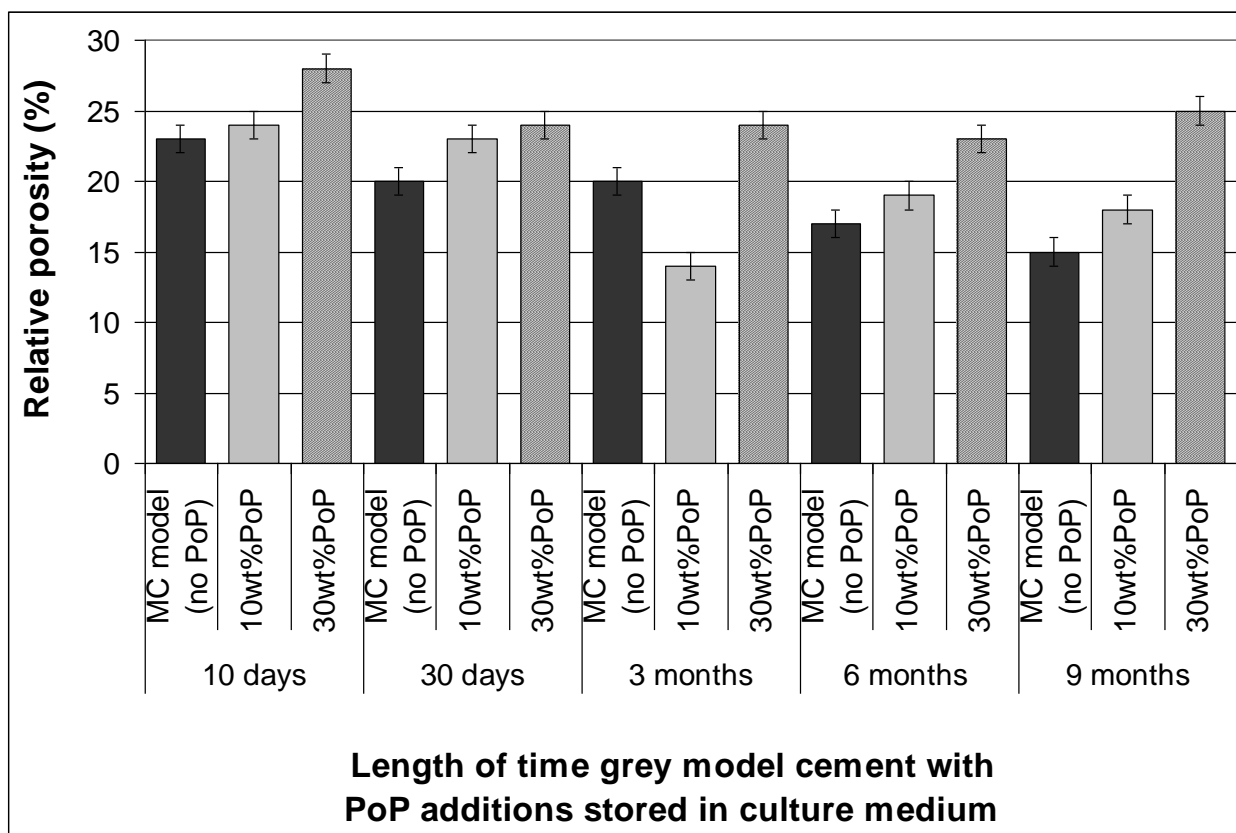


Figure 48: Bar chart to represent the RP of cements stored in culture medium with or without PoP additions. Error bars represent the approximate error of the method combined with the error attached to calculating the RP ($\pm 1\%$). Grey model cements without PoP and cement containing 10 wt% PoP had RP that generally decreased with time, though the cements containing PoP had higher RP when compared with cements without PoP.

6.1.4 Effect of storage in medium on CS and RP of modified white model cements

The CS of white cements did not change significantly with increased storage time in culture medium which was similar to the trend for CS of white cements stored in distilled water (figures 45 and 49). White model cements containing 10 wt% PoP had the highest CS at any time point when compared with unmodified cements and 30 wt% PoP-containing cements (figure 49). RP of cements modified and unmodified with 10 wt% PoP decreased with storage time in culture medium, however, remained similar for cements that contained 30 wt% PoP (figure 50).

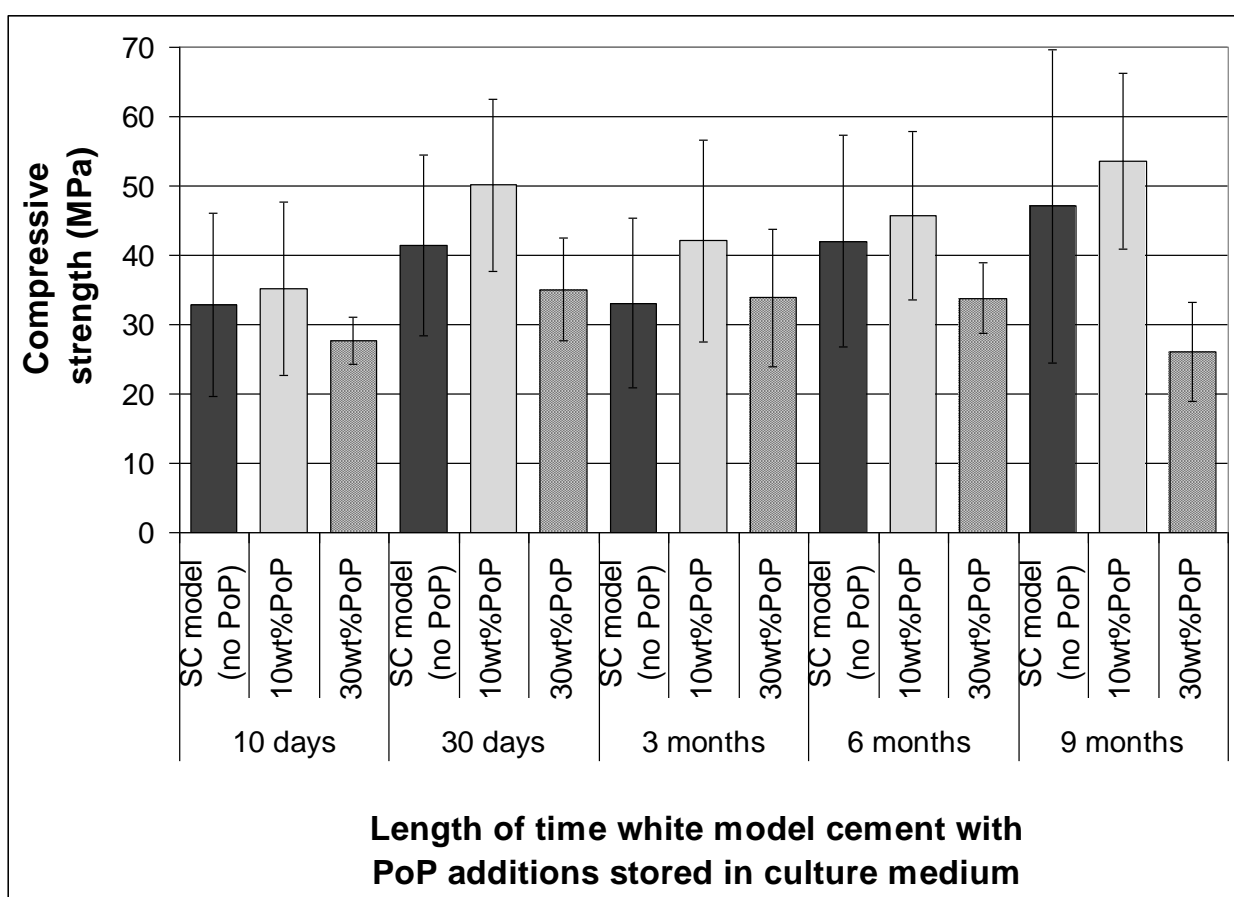


Figure 3: CS of cements with or without PoP that have been stored in culture medium for up to 9 months. Error bars on the graph represent the standard deviations for mean values that were plotted. Culture medium storage of 10 wt% PoP-containing cement had higher CS with storage time when compared with other cements stored in culture medium.

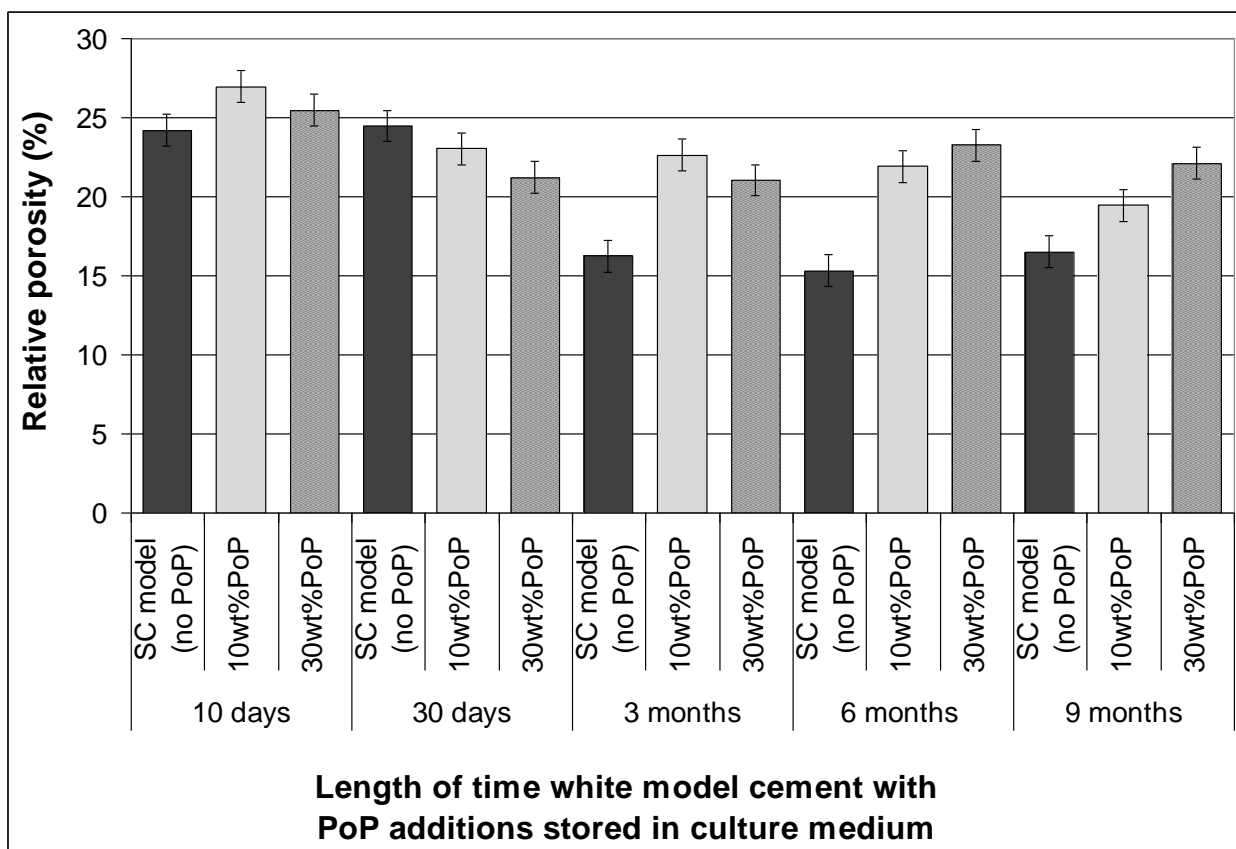


Figure 50: RP of cements with or without PoP additions that had been stored in culture medium for up to 9 months. Error bars represent the approximate error of the method and calculations used to retrieve RP ($\pm 1\%$). RP varied according to composition, however, the white SC model cements without PoP had the lowest RP when compared with cements containing 10 or 30 wt% PoP.

Cements with no PoP added which were stored for 10 days had the highest dry density compared with cements with PoP additions. Densities were observed to be higher for cements set for 30 days that contained PoP additions. However there was a decreased density observed for the model cement that did not contain additives ($1.94 \pm 0.01 \text{ g/cm}^3$). Density increased for cement containing no additions and 10 wt% PoP additions which had been stored for 3 and 6 months but then decreased when set for 9 months in culture medium for cements without additives. On the contrary, dry densities of cements with 30 wt% PoP additions decreased when stored between 3 and 9 months.

6.2 *Long term solubility of the model system with PoP*

6.2.1 **Release of calcium ions from cement into water**

The release of calcium ions was greatest in water solutions that contained cements modified with 30 wt% PoP after 1 week compared with model cements and the 10 wt% PoP modification, see figure 5. After week 2, the concentration of calcium ions in water solutions containing model cements with or without PoP continued to increase until week 4, until concentrations of calcium ions released reached the highest concentration of 0.036 M. The concentration of calcium ions release from PoP modified and unmodified cements decreased at 5 and 6 weeks to less than half the concentration of calcium ions measured at week 4 but began to gradually increase between weeks 7 and 10 (see figure 51). The release of calcium ions declined further during the 11th week and continued to decline until the concentration plateau between weeks 17 and 52, when the concentrations of calcium ion release remained low (<0.002 M) for PoP-modified and unmodified cements, see figure 51.

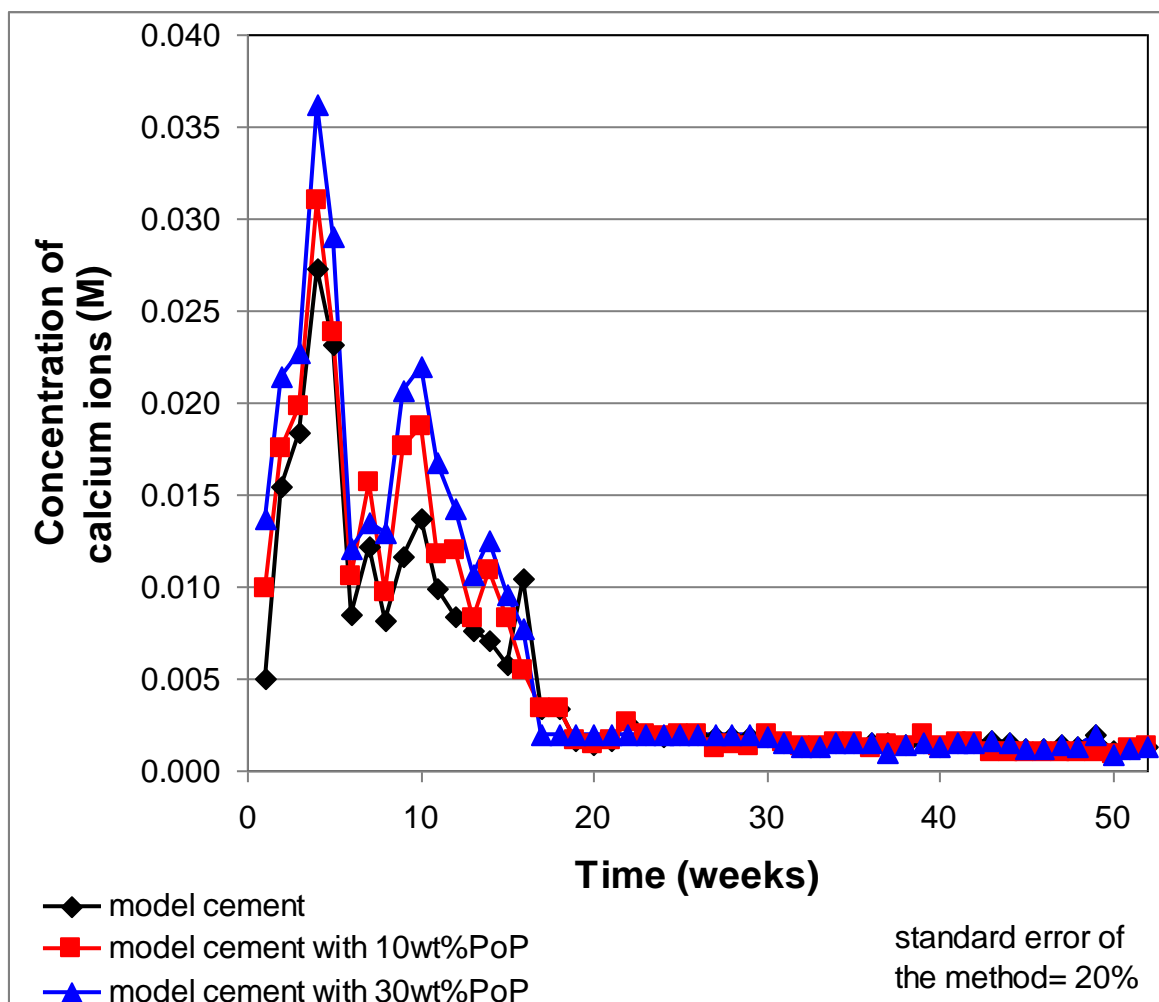


Figure 51: The release of calcium ions (M) from model cements with or without PoP additions into water (which cements were stored within) with time (weeks). The estimated approximate error of the method used to obtain the data plotted was $\pm 20\%$. Approximate errors were not plotted on the graph as error bars so that plot points may be seen more clearly. There were more calcium ions released from cements with or without PoP additions into water, when measured in week 4.

The pH of the water in which model cements (with or without PoP) were stored, remained high throughout the study. The pH of storage solutions containing cements with or without PoP additions was 12.3 after 1 week of storage and decreased only slightly to pH 11.2 after 52 weeks of storage (figure 51).

6.2.2 SEM analysis of modified grey model cements

6.2.2.1 *Cements stored in distilled water*

Little difference was observed between the microstructures of the fracture surfaces of grey model cements with or without PoP additions that had been stored in water for 10 days (figure 52). The outer surface of 30 wt% PoP-containing cement was very similar to the appearance of the fracture surface (see figure 52, images E and F). A smooth surface that consisted of tightly compacted particles was not observed for the outer surface of the model cement containing 30 wt% PoP, but was observed on the outer surface of the model cement and 10 wt% PoP-containing cement (figure 52, images A and B). More pores were observed on the outer surface of 30 wt% PoP-containing cement after 10 days of storage in water, when compared with the porosity observed for this cement when viewing the microstructure of the fracture and outer surfaces of the model cements with or without 10 wt% PoP, after 10 days water storage.

After 9 months of storage, the outer surface of the model cement differed when compared with cements containing PoP additions since the particles on the surface did not appear to be as compact for the model cement as 10 and 30 wt% PoP-containing cements (figure 53, image A-C) and cracks were apparent on the fracture surface of 10 wt% PoP-modified cement unlike model cement (figure 53, images B and D).

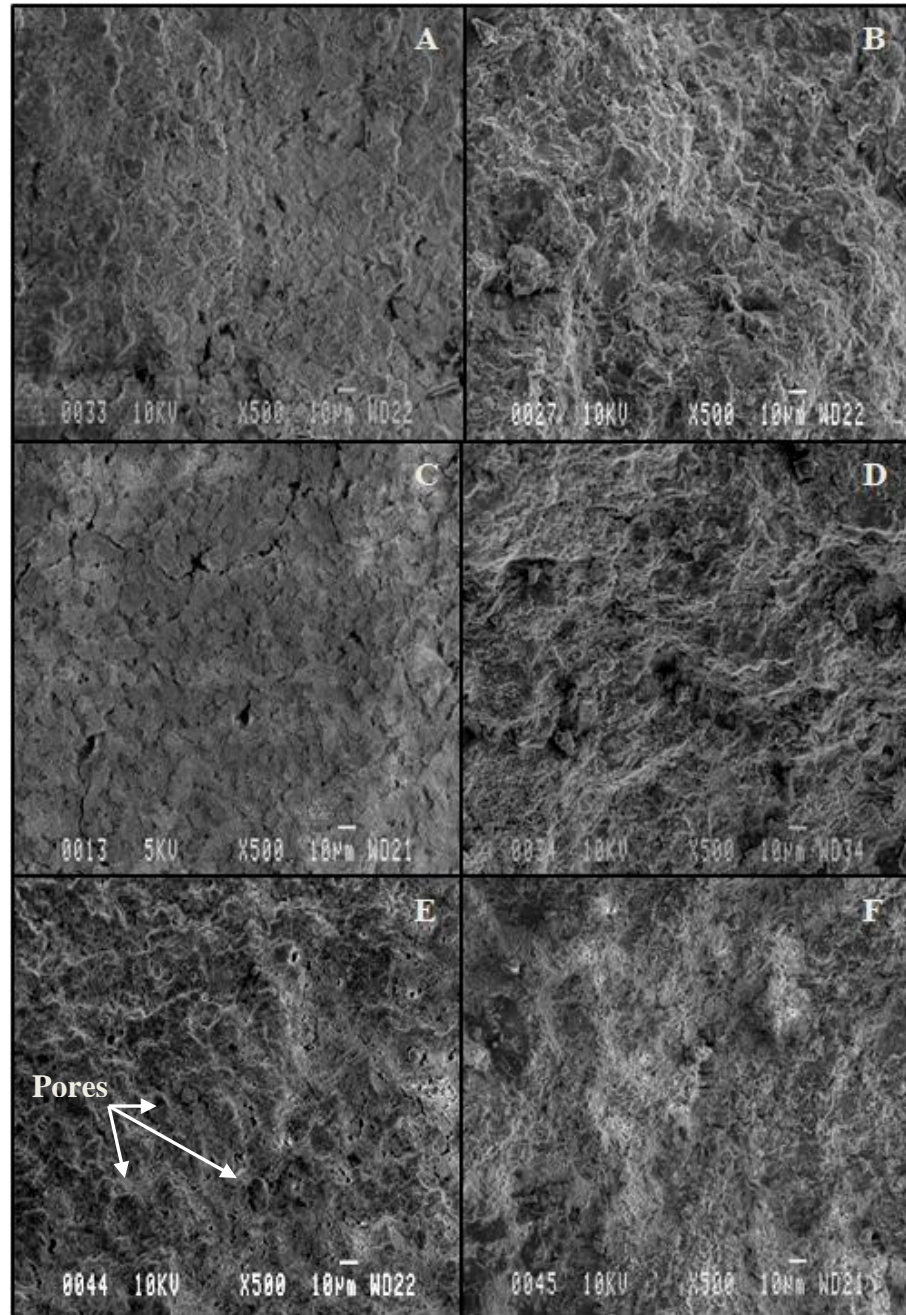


Figure 52: SEM photomicrographs of the outer and fracture surfaces of the grey model cement with or without PoP additions stored in water for 10 days. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images to the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. More pores were apparent in the outer surface of 30 wt% PoP-containing grey model cement compared with unmodified grey model cement and 10 wt% PoP containing grey cement.

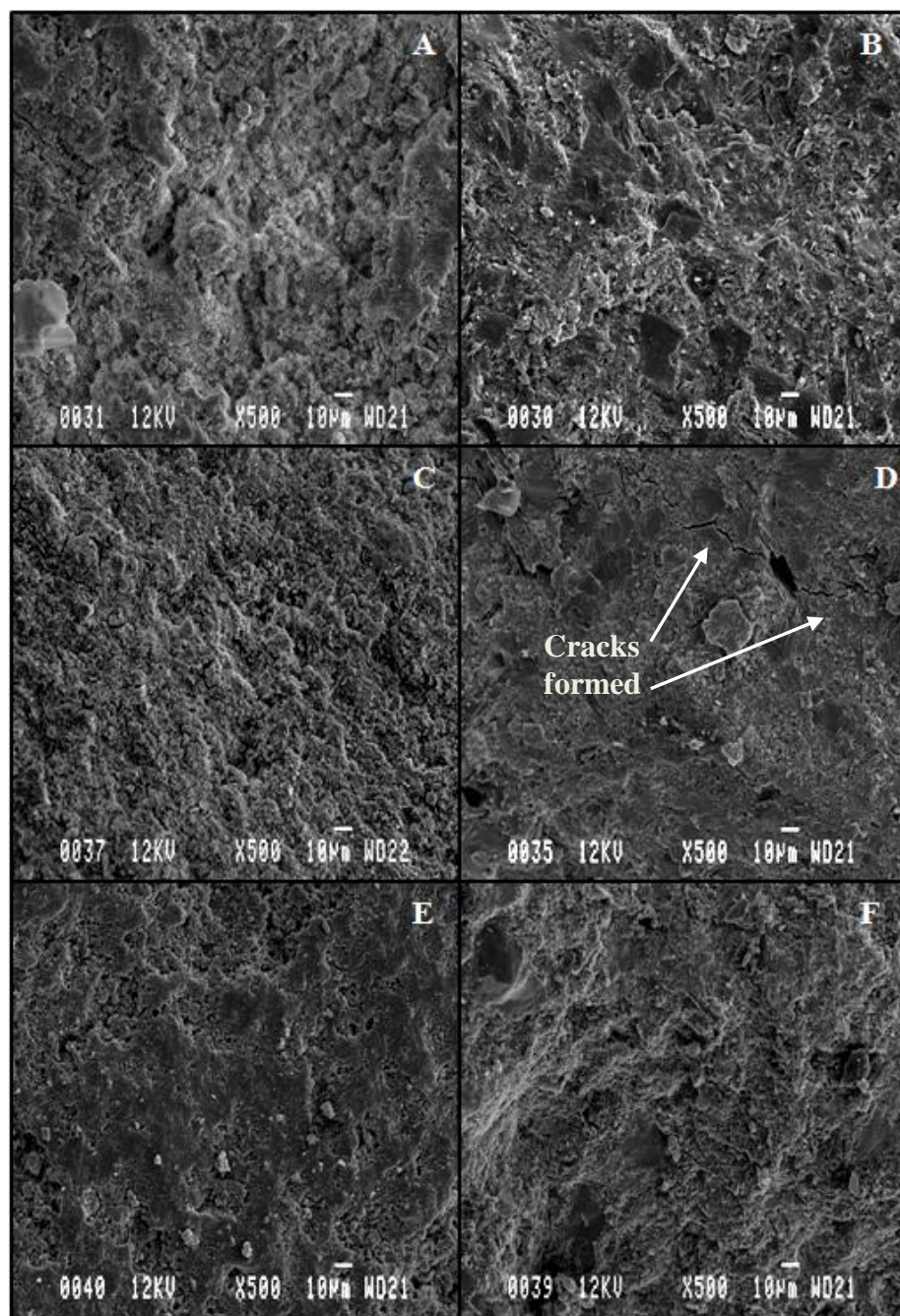


Figure 53: SEM photomicrographs of grey model cements stored in water for 9 months. Images to the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images to the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. The fracture surfaces of PoP-modified grey cements appeared to be denser than unmodified model cements.

RESULTS

The outer surface of the model cement resembled that of the fracture surface of the model cement since the surface did not have tightly compacted particles but more cement plates that were fused together with small needle-like structures and small cement particles covering the plates (figure 54).

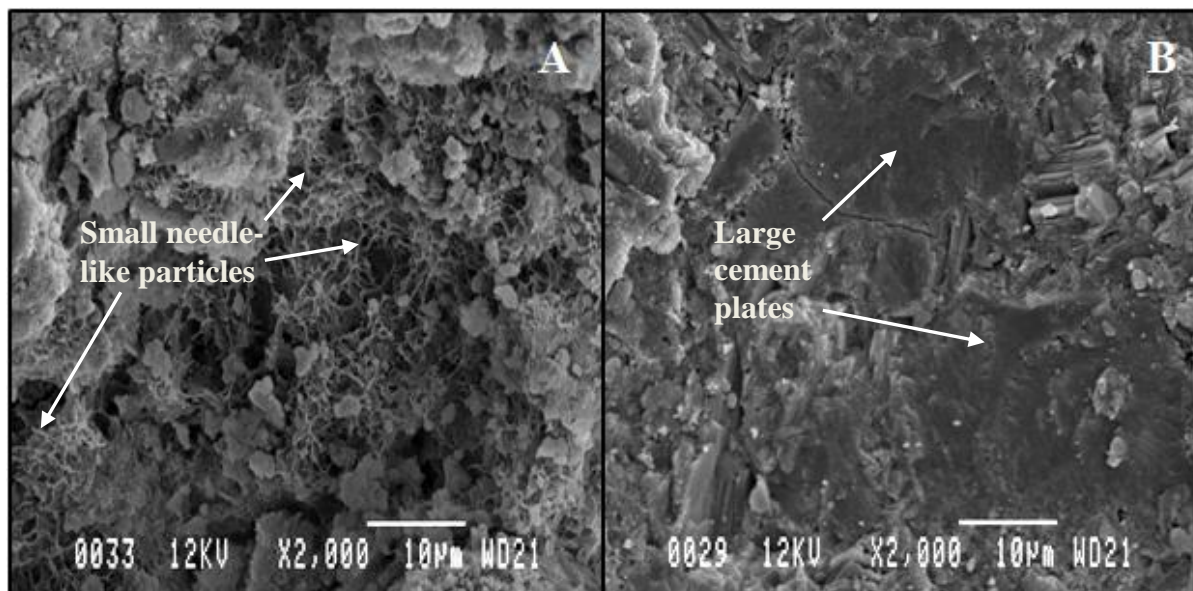


Figure 54: SEM photomicrographs of the outer (A) and fracture (B) surfaces of MC model cements stored in water for 9 months. Large cement plates were evident on the fracture surface at x 2000 magnification, however, small needle-like particles were observed on the outer surface of the model cement.

The fracture surfaces of cements containing 10 and 30 wt% PoP were relatively similar since both consisted of large cement plates covered by smaller cement particles, however, crack propagation was more apparent for cements consisting of the model cement with 10 wt% PoP (figure 53, images B, D and F).

Grey modified and unmodified cements that were stored in water for 10 days had a denser outer surface than the outer surface of 9 month-stored cements (figures 52 and 53). Pores were more visible on the outer surfaces of cements that did or did not contain PoP stored for 9 months compared with cements stored for 10 days (figure 52 and 53). However, no needle-shaped

cement particles were observed upon the outer surfaces of cements stored in water for 10 days (figure 53), though these needle-like structures were evident in SEMs of the model cement stored for 9 months only (see figure 54).

6.2.2.2 *Cements stored in culture medium*

The outer surface of the grey MC model cement appeared to be similar to the fracture surface of the model cement since the surface was uneven but had a more compact cement structure (figure 55, images A and B) compared with 10 and 30 wt% PoP-modified cements that were less dense, due to increased PoP content. Pores were evident in cements containing PoP additions; however, 10 wt% PoP-containing cements had fewer pores when the fracture surfaces were examined using SEM compared with the fractures surfaces of 30 wt% PoP-modified cements (figure 55).

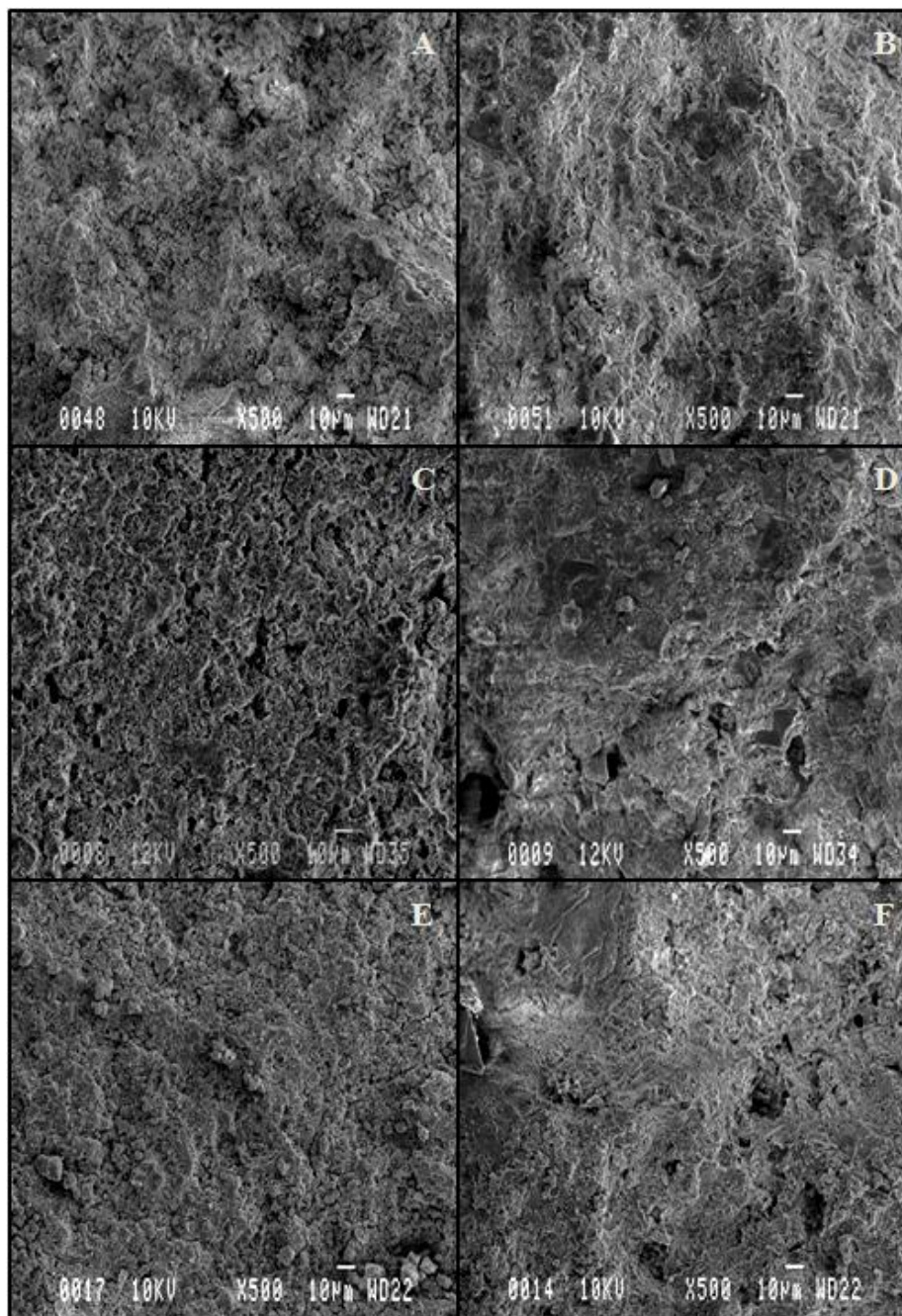


Figure 55: SEM photomicrographs of the outer and fracture surfaces of the MC model cement with or without PoP additions stored in culture medium for 10 days. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Cement structures were less compact when PoP content was increased.

RESULTS

No differences were observed between grey model cements stored in culture medium for 10 days when compared with those stored in culture medium for 9 months. However, cements containing 10 wt% PoP after 9 months of storage had larger, more rounded particles ($>10\text{ }\mu\text{m}$) formed upon the outer surface which differed from the outer surfaces of unmodified model cements stored for 9 month in culture medium (figure 56, images A and C) and 10 wt% PoP-modified cements after 10 days of storage, which contained smaller ($<10\text{ }\mu\text{m}$) particles (figures 55 and 56, images labelled C). 30 wt% PoP-containing cements stored in culture medium for 9 months had small particles apparent on the outer surface which were not present on the cements that were stored for only 10 days in culture medium (figures 55 and 56, images labelled E).

There were noticeable differences between the outer and fracture surfaces of cements that had been stored in culture medium for 10 days and 9 months (figure 55 and 56) since at 9 months cements looked to be denser on the fracture surface compared with the outer surfaces of cements. However, pores and crack propagation were apparent on the fracture surfaces of 10 and 30 wt% PoP-containing cements (figure 56, image D and F) but were not so prevalent after 10 days of culture medium storage (figure 55, images D and F) and were not present in the fracture surface of the model cement after 9 months of culture medium storage (figure 56, image B).

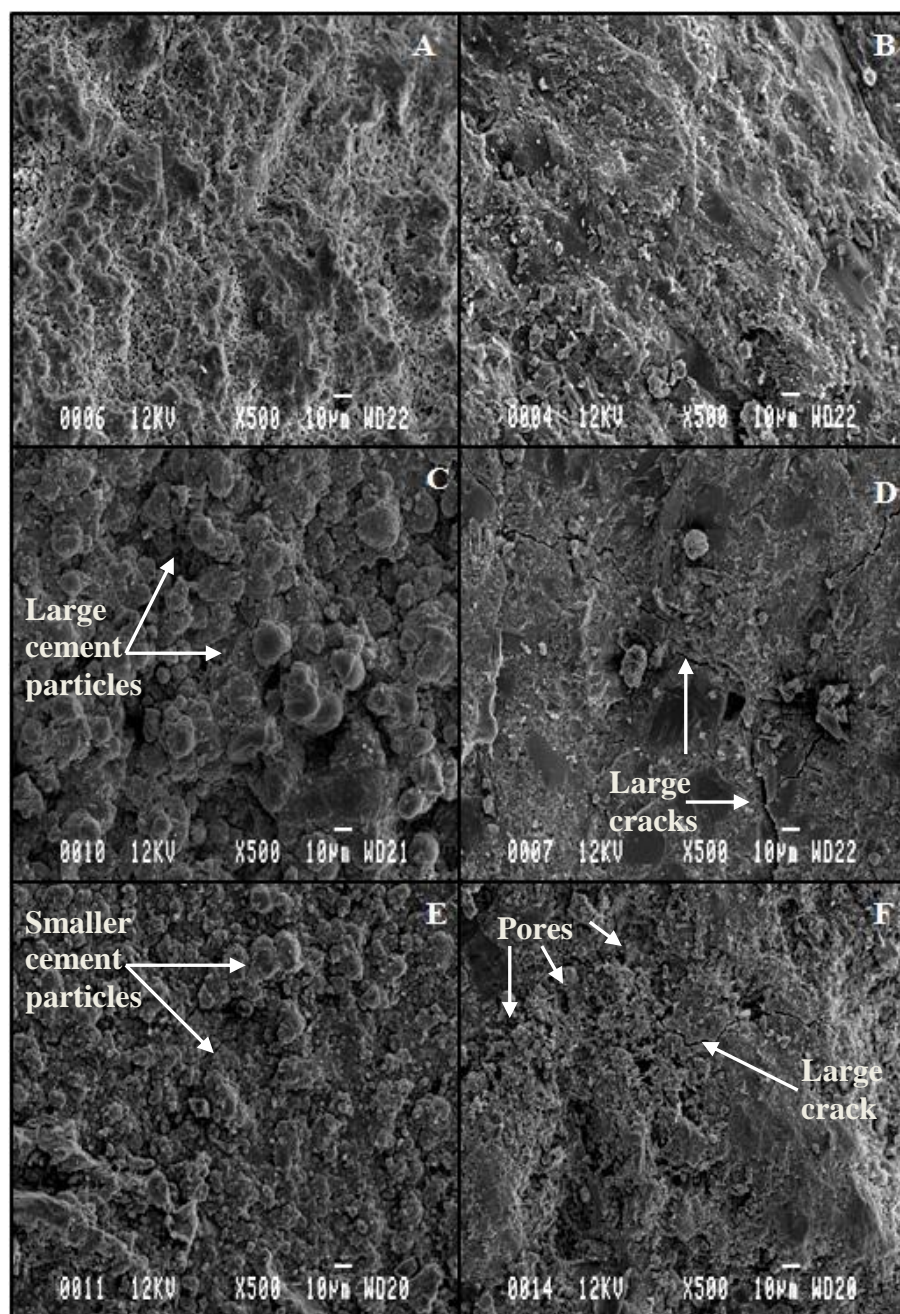


Figure 56: SEM photomicrographs of the outer and fracture surfaces of the MC model cement with or without PoP additions stored in culture medium for 9 months. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Density decreased with increased PoP content to grey model cements and cracks were apparent in PoP-modified grey cements.

6.2.3 SEM analysis of modified white model cements

6.2.3.1 *Cements stored in distilled water*

The white SC model containing 10 wt% PoP had a more dense outer structure when compared with the structure of the outer surface of the model cement with no PoP additions (figure 57, images A and C). The fracture surfaces appeared to be similar for white model cements with or without PoP additions, stored in water for 10 days as MC model cements with or without PoP which were also stored for 10 days in distilled water (figures 52 and 57, images B, D and F).

However, with prolonged storage in water, the outer surface of the white model cement began to alter since more porosity was evident (figure 58, image A) after 9 months when compared with 10 days of storage in water (figure 57, image A). Pores were present for cements containing 10 wt% PoP stored for 9 months in water, however, these were much smaller than those apparent in the structures of cement containing 30 wt% PoP additions (figure 58, images C and E). The fracture surfaces of white model cements with or without PoP remained similar to those observed for MC model cements with or without PoP after 9 months of water storage (figures 53 and 58, images labelled B, D and F), however, there were no cracks apparent in 10 wt% PoP-containing white model cements (figure 53, image D) and when compared with 10 wt% PoP-containing MC model cements (figure 58, image D). Fracture surfaces of 30 wt% PoP-modified cements had some gaps within the structure which could have been left from dislodged particles (figure 58, image F).

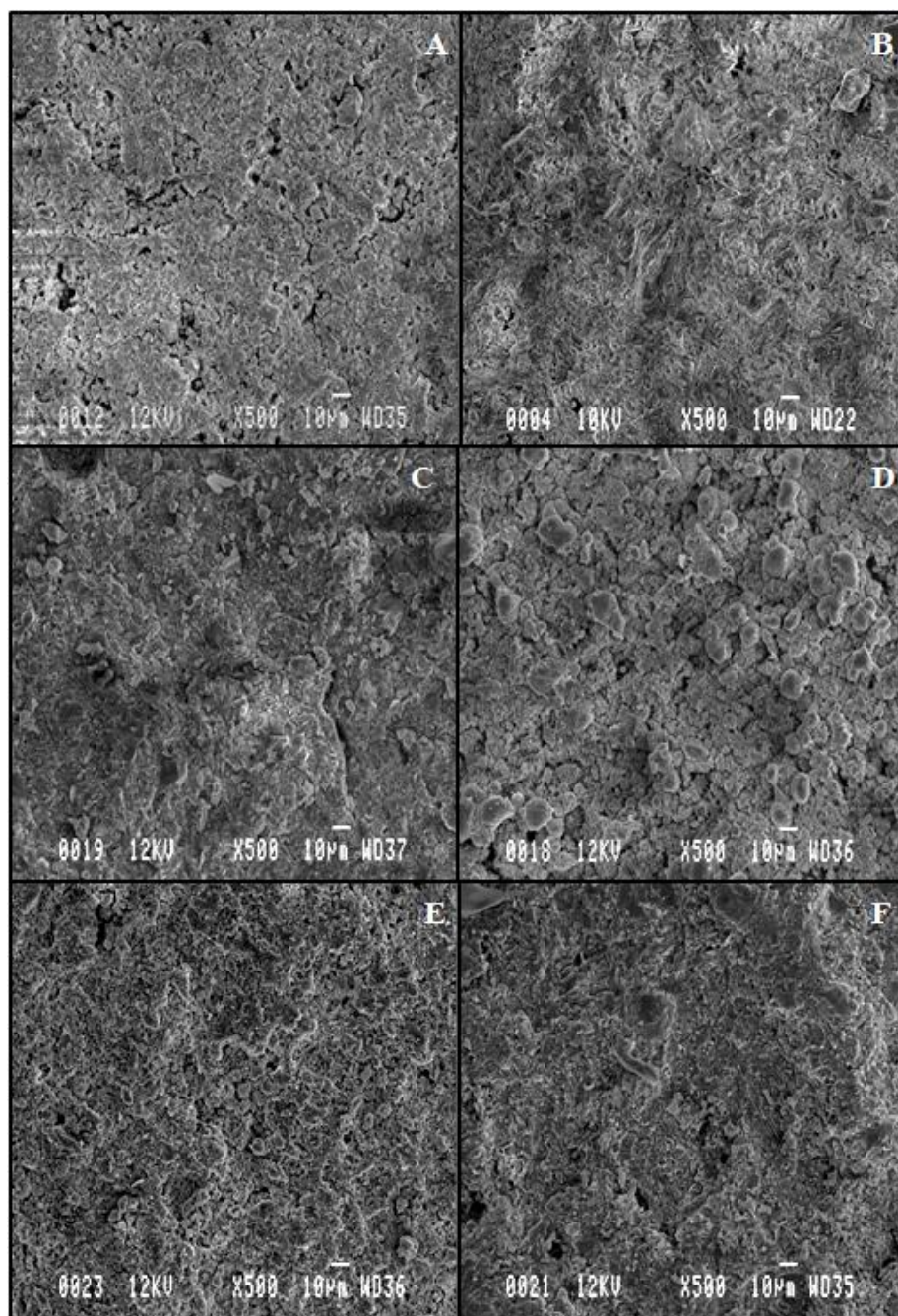


Figure 57: SEM photomicrographs of the outer and fracture surfaces of the white SC model cement with or without PoP additions stored in water for 10 days. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Outer and fracture surfaces were similar in appearance regardless of cement composition.

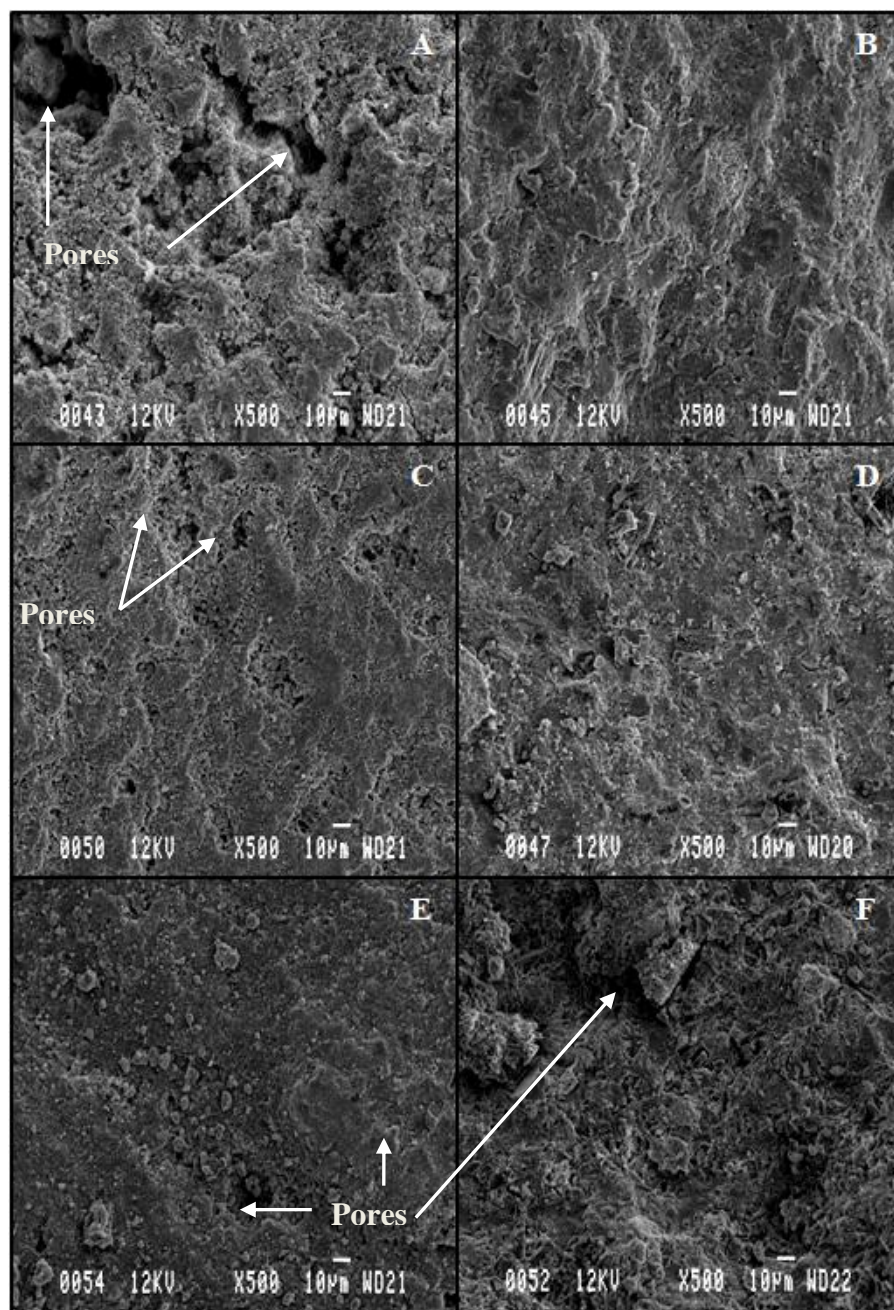


Figure 58: SEM photomicrographs of the outer and fracture surfaces of the white SC model cement with or without PoP additions stored in water for 9 months. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Fracture surfaces were less porous compared with outer surfaces of unmodified and PoP-modified cements.

6.2.3.2 *Cements stored in culture medium*

The outer surface of white SC model cements stored for 10 days in culture medium was similar to the same cement that had been stored in distilled water for the same period of time. The outer surface of model cement containing 10 wt% PoP additions stored for 10 days in culture medium consisted of small rounded clusters of cement particles which was not apparent in cements containing 30 wt% PoP or the model cement only (figure 59, images A-C). Pores were apparent on the outer surface of 30 wt% PoP-modified white cements that had been stored in culture medium for 10 days (figure 59, images E and F). The fracture surfaces of cement with or without 10 wt% PoP which was stored in culture medium for 10 days did not differ much since the fracture surfaces consisted of large cement plates fused together (figure 59, images B and D). Pores and gaps within the structure which could have been left from dislodged particles were present on the fracture surfaces of 30 wt% PoP-containing cement (figure 58, image F).

Rounded clusters of cement particles were evident on the outer surfaces of cements with or without PoP additions that had been stored for 9 months (figure 60, images A, C and E), however, there were fewer clusters present on the surface of 10 wt% PoP-containing cement and those present upon the model cement without PoP were less regular in size and shape (figure 60, image A) when compared with cement with 30 wt% PoP additions (figure 60, image C). The fracture surfaces did not differ much from those apparent in cements that had been stored in culture medium for 10 days but fracture surfaces were denser than the outer surfaces of cements stored in culture medium for 9 months (figure 60).

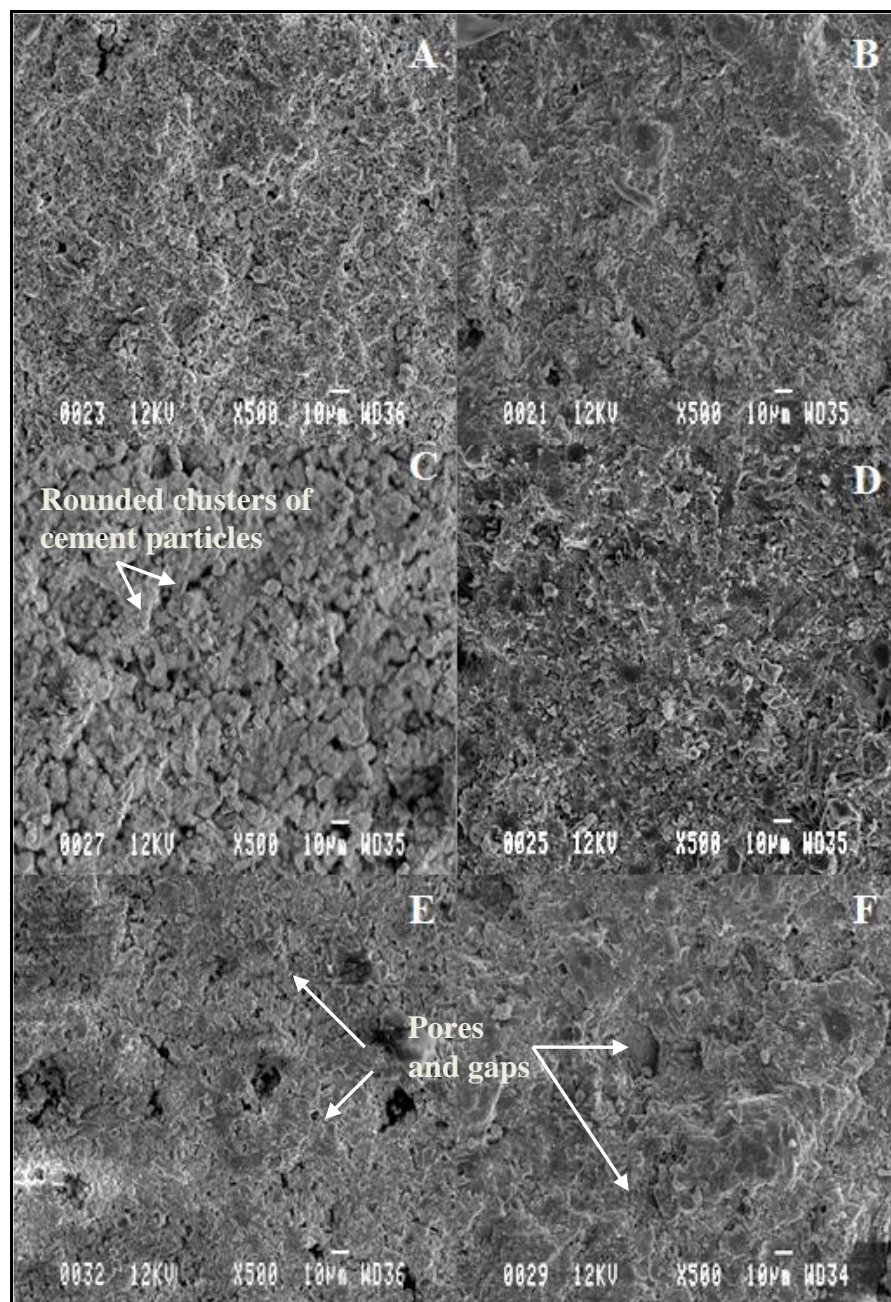


Figure 59: SEM photomicrographs of the outer and fracture surfaces of PoP-modified white SC model cement stored in culture medium for 10 days. Images on the left (A, C and E) represent the outer surfaces of unmodified and 10 or 30 wt% PoP-modified model cement, respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. More pores and gaps were present in the fracture and outer surfaces of 30 wt% PoP-modified cements.

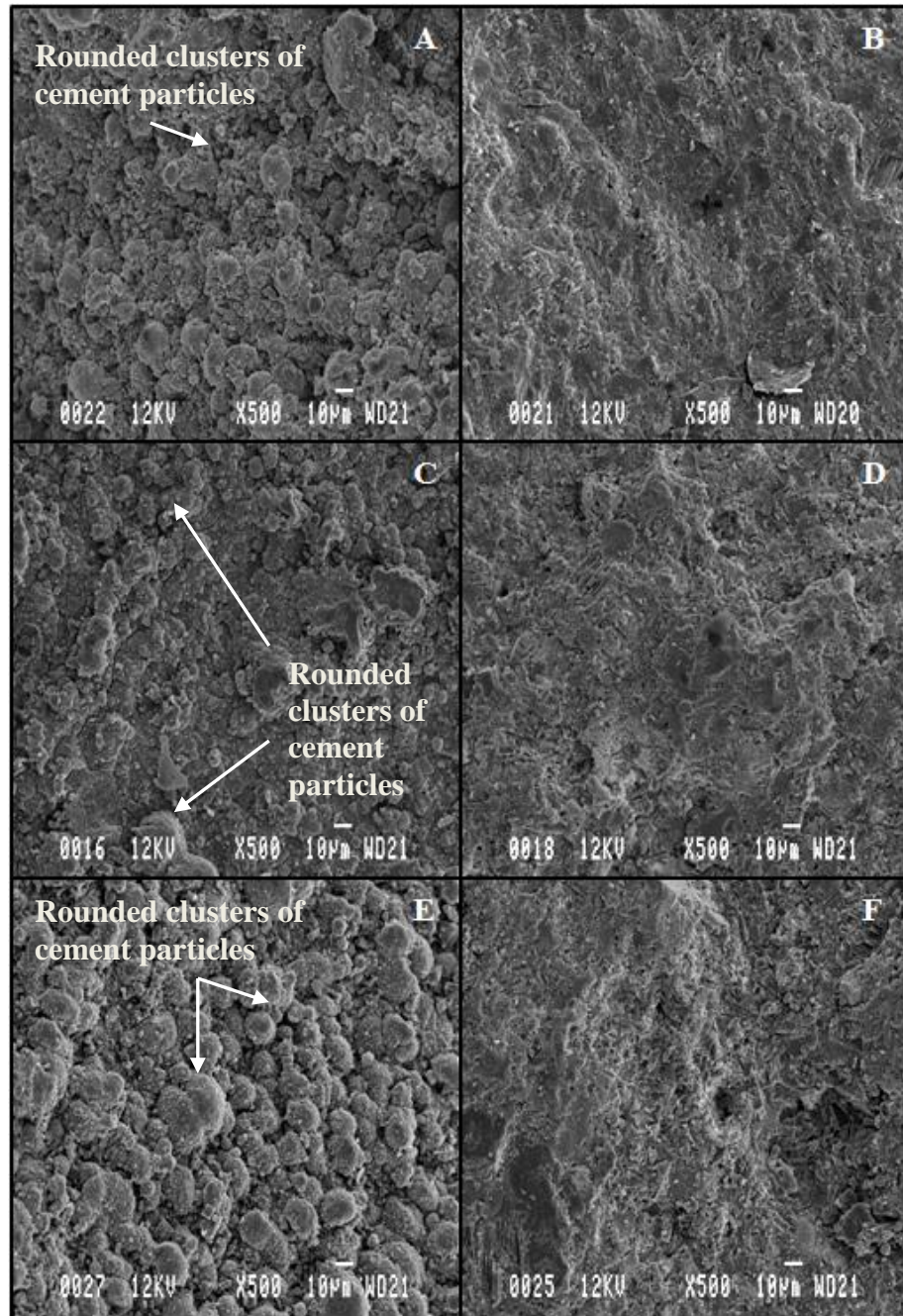


Figure 60: SEM photomicrographs of the outer and fracture surfaces of the white SC model cement with or without PoP additions stored in culture medium for 9 months. Images on the left (A, C and E) represent the outer surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Images on the right (B, D and F) are representative of the fracture surfaces of the model cement, model cement containing 10 and 30 wt% PoP respectively. Fracture surfaces were denser than the outer surfaces of cements, which consisted of rounded clusters of cement particles rather than cement plates.

7 In vitro study of model cement and MTA containing PoP

7.1 *Periosteal cultures containing modified grey model cements*

A typical cell growth curve was apparent for control cultures. Cell densities were low for all cultures up to 4days of culturing demonstrating little cell proliferation but after this, cell densities increased significantly with time ($p<0.05$). Periosteal control cultures that did not contain cements proliferated significantly more ($p<0.05$) than cement containing cultures after 6 days of culturing until the end of the experimental period (see figure 61). Cultures containing model cements with or without PoP additions revealed significantly lower cell densities compared with control cultures that consisted of periosteal cells only at 8 days until the end of the experimental period of 14days ($p<0.05$). Cultures containing PoP-modified and unmodified model cements had significantly greater cell densities at 8 days when compared with the density of periosteal cultures at 6 days. Despite the increased cell density recorded at 8 days compared with at 6 days, cultures containing 30 wt% PoP and unmodified model cements showed statistically similar cell counts (see figure 61).

Cell proliferation continued at 12 days for periosteal cultures with or without cements. However, after 14days cell proliferation ceased to continue for cultures containing model cements and cements containing 30 wt% PoP and similar cell densities to those taken at 12 days were measured. Cell proliferation began to decrease between 12 and 14days for control cultures, seen in figure 61, but remained statistically higher than cultures containing model cements and PoP-modified cements ($p<0.05$).

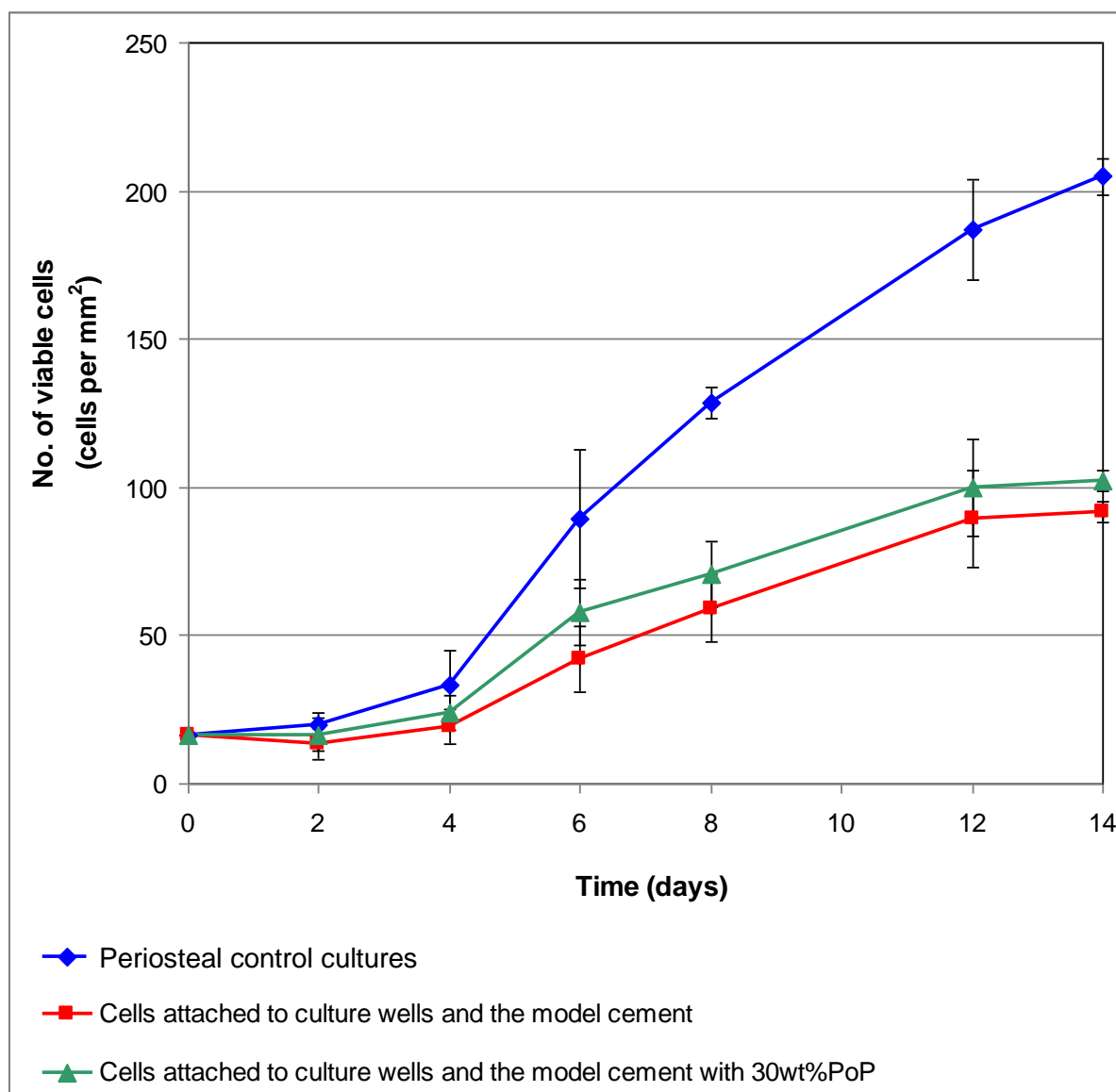


Figure 61: The cell densities (total from culture well/ culture well and cement) of periosteal cultures containing cement samples with or without PoP with time (14days) No cells attached to cements with or without PoP. Similar cell densities were observed for cultures containing model cements and cements with PoP additions, but significantly higher for control cultures ($p < 0.05$). Error bars represent the standard deviation of the mean values.

7.2 Osteoblastic cultures containing modified grey model cements

Osteoblast cell densities decreased with the addition of both PoP-modified and unmodified cements relative to control cultures, see figure 62, throughout the entire experiment. At 4 days, the rate of cell proliferation was greater than that for all cultures at 2 days.

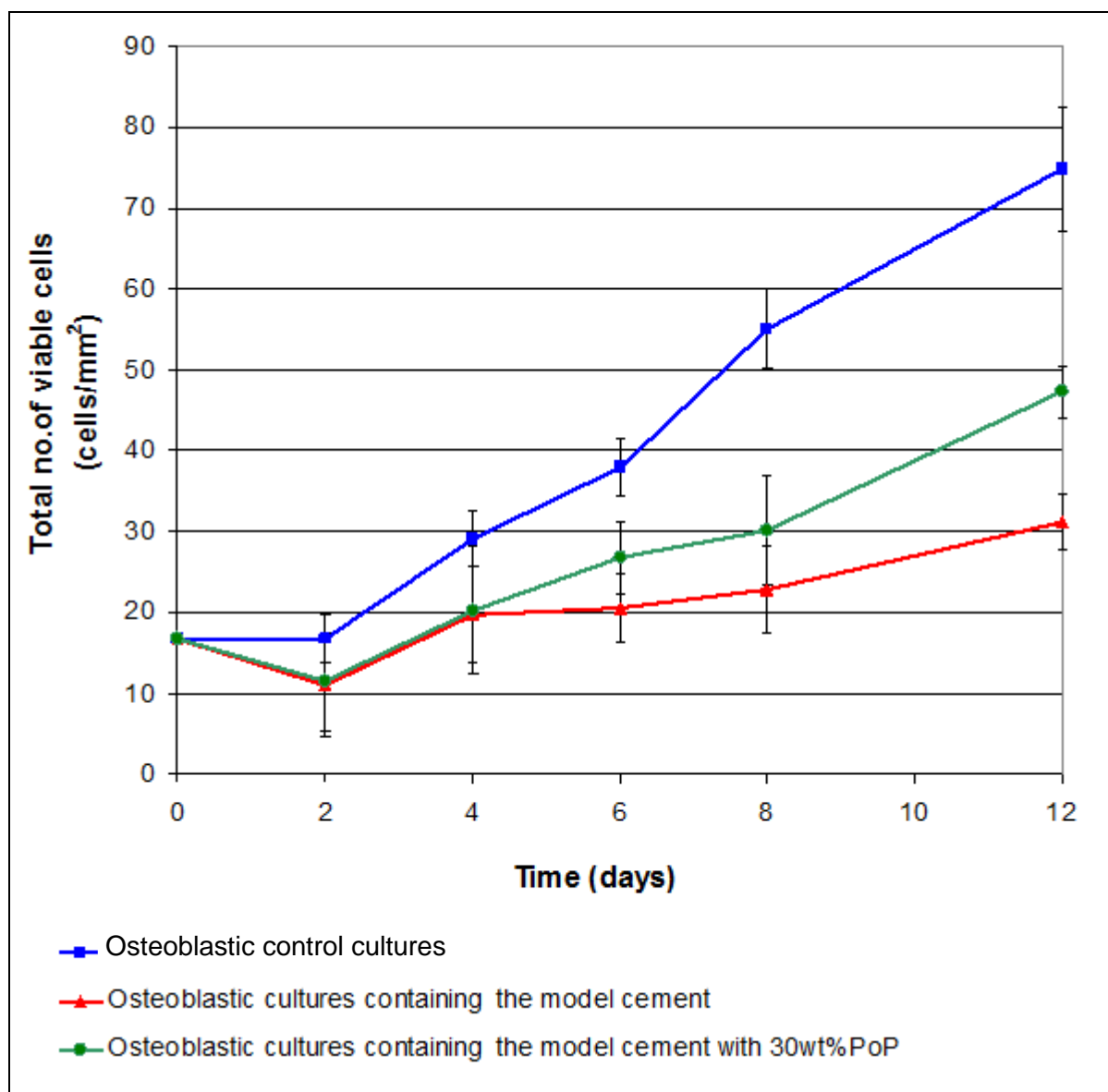


Figure 62: Density of viable osteoblasts attached to cell culture wells (and cells attached to cement) with or without model cements that had or had not been modified with PoP. Cultures containing cements modified with or without 30 wt% PoP had lower cell counts when compared with cultures containing just primary osteoblasts.

Cell proliferation increased significantly for control cultures from day 6 to day 12 but increased at a slower rate for cultures with 30 wt% PoP-containing cements and to a lesser extent for cultures with model cements ($p<0.05$), see figure 62. Cell density was highest for control cultures throughout the experimental period.

However, addition of PoP-modified cements to cultures caused cell densities to increase significantly more after 12 days compared with cultures containing model cements without PoP ($p<0.05$) see figure 62.

7.3 *Neonatal BMSC cultures containing modified grey model cements*

7.3.1 **Cells seeded onto cement in 4 ml of culture medium**

Cell densities decreased after 2 days for cultures containing the model cement with or without PoP and the neonatal BMSC control cultures but to a lesser extent for the latter (see figure 63). Cell densities remained low between 4 and 8 days for cultures containing cements with or without PoP. Neonatal BMSC control cultures had a significantly higher rate of cell proliferation when compared with cultures containing cements ($p<0.05$). After 8 days, the rate of cell proliferation increased significantly ($p<0.05$) for all cultures (see figure 63). After 14 days, cultures containing model cement exhibited the lowest cell density compared with PoP-containing cultures whilst control cultures were significantly higher ($p<0.05$).

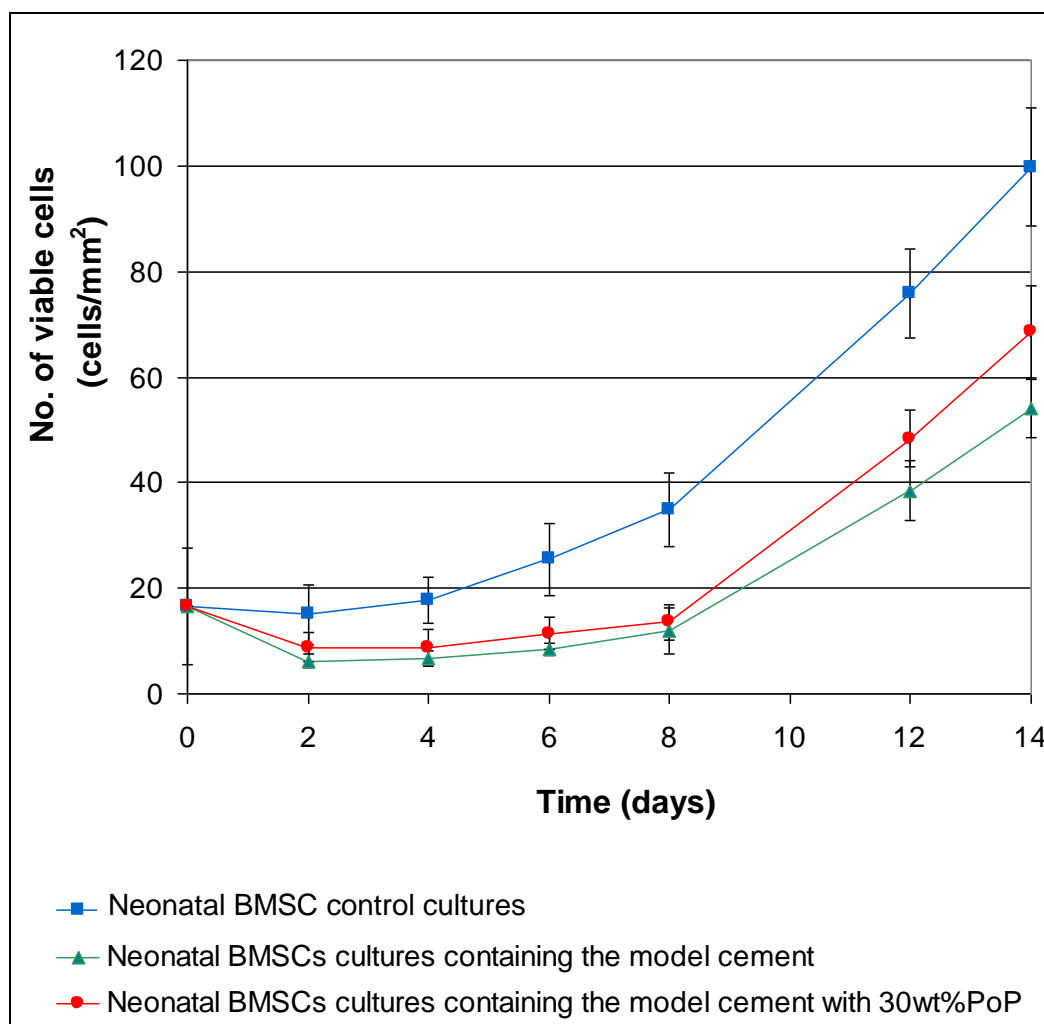


Figure 63: Total number of viable neonatal BMSCs (seeded directly onto cements in 4 ml of medium) attached to cell culture wells (and cells attached to cement) with or without model cements and PoP modified model cement. Control neonatal BMSC cultures had significantly higher ($p < 0.05$) cell densities compared with cultures containing model cements with or without PoP, by the end of the experimental period of 14 days. Cell densities for cultures containing cements were similar throughout the experimental period.

7.3.2 Cells seeded onto cements in 50 μ l of culture medium

In a similar manner to cultures where cells were seeded directly with the total volume of culture medium, cells seeded directly in a small volume of medium onto cements in cultures showed decreased cell densities after 2 days compared with the control. Cell densities of model cements and 30 wt% PoP-containing cement cultures for 4 to 8 days remained similar to the cell

RESULTS

densities at 2 days (figure 64) but by 10 days, no cells had attached to cements or culture wells containing these cements. On the contrary, cell density increased significantly for control cultures which was significantly more than the cell densities of cultures that contained model cements and cements with 30 wt% PoP throughout the experimental period of 10 days ($p < 0.05$), see figure 64.

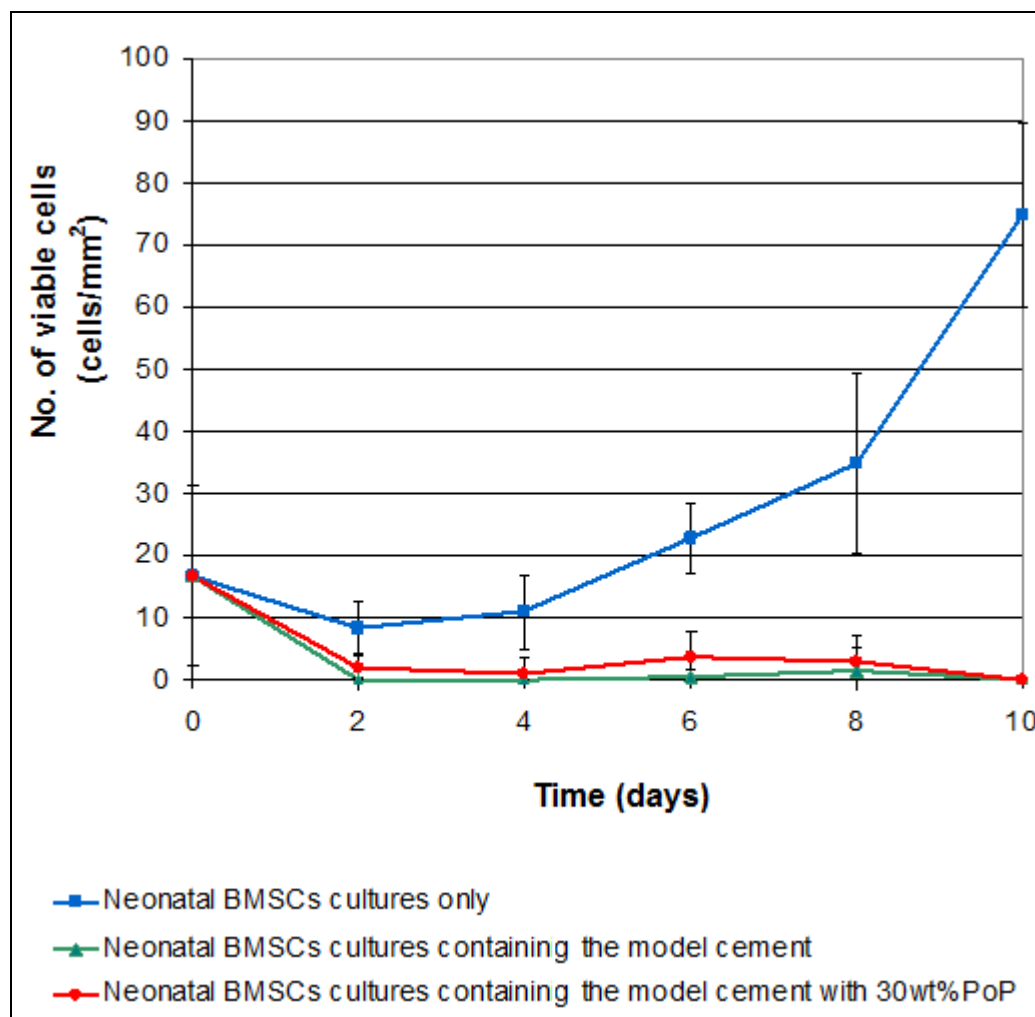


Figure 64: Total number of viable neonatal BMSCs (seeded directly onto cements in 50 μ l of medium and cultured for an hour) counted in cultures that contain cements with different compositions after being cultured for up to 10 days. The graph showed that cultures containing model cements with or without 30 wt% PoP had significantly lower densities compared with control cultures that consisted of neonatal BMSCs only.

Neonatal BMSCs demonstrated similar proliferation patterns during this experiment as observed for the first experiment where cells had been seeded into culture wells in a larger

volume of culture medium but the number of viable cells was lower for cells seeded into wells in a small volume of medium (figure 64) when compared with cells seeded into wells in larger volumes (see figure 63).

7.3.3 Cements placed into established cell cultures

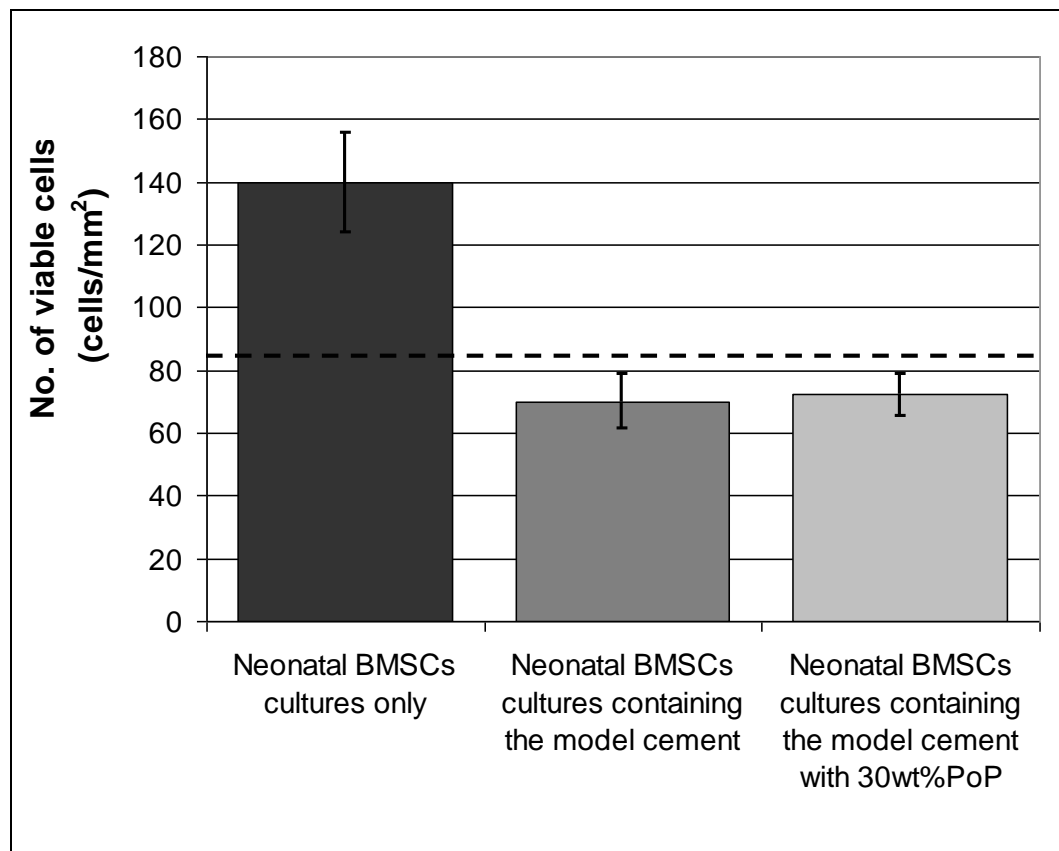


Figure 65: Cell densities of viable neonatal BMSCs counted after 3 days in established cultures that contained the model cement with or without PoP additions cultured. The dashed line represents the cell density seeded into culture wells at the beginning of the experiment. The combined density of cells attached to culture wells and cements was recorded. Cultures that contained PoP-modified or unmodified model cements had significantly lower cell densities recorded compared with control cultures.

Densities of neonatal BMSCs attached to both culture wells and cements decreased considerably following the addition of cements with or without PoP additions (figure 65).

7.4 *Adult BMSC cultures containing modified model cements and MTA*

7.4.1 **Cells seeded into 4 ml of culture medium**

Control cultures that consisted of adult BMSCs only, model cement-containing cultures and cultures containing cements with 30 wt% PoP had decreased cell densities at 2 days compared with the initial seeding density but cultures containing model cements had the lowest cell density as shown in figure 66. Cells in cultures containing PoP-modified cements demonstrated greater viability (80%) compared with cells in model cement-containing cultures (72 %) but the cell viability was greatest when cultures did not contain any cements (100%) and remained high for control cultures throughout the study of 14days (100%). No cell attachment was observed to cement samples with or without PoP additions at 2 days in culture.

Cell proliferation was observed for all cultures with or without model cements from 4days until the end of the experimental period of 14days, but the rate of cell proliferation was lowest for cement containing adult BMSC cultures (figure 66). Cell viabilities mirrored patterns of cell densities for cultures containing cements with or without PoP. Cells proliferated further for cultures containing adult BMSCs only to 45 ± 7 cells per mm^2 at 6 days, which was over 3 times the number of viable cells counted surrounding model cements and more than double the viable cell density of cultures containing model cements with 30 wt% PoP additions as shown in figure 66.

The density of adult BMSC control cultures increased significantly at 10 days ($p < 0.05$), compared with cell densities of containing cultures PoP-modified and unmodified model cements which remained similar. Cell viability increased at 10 days for adult BMSC cultures containing model cements with or without 30 wt% PoP to 100 and 99 % respectively.

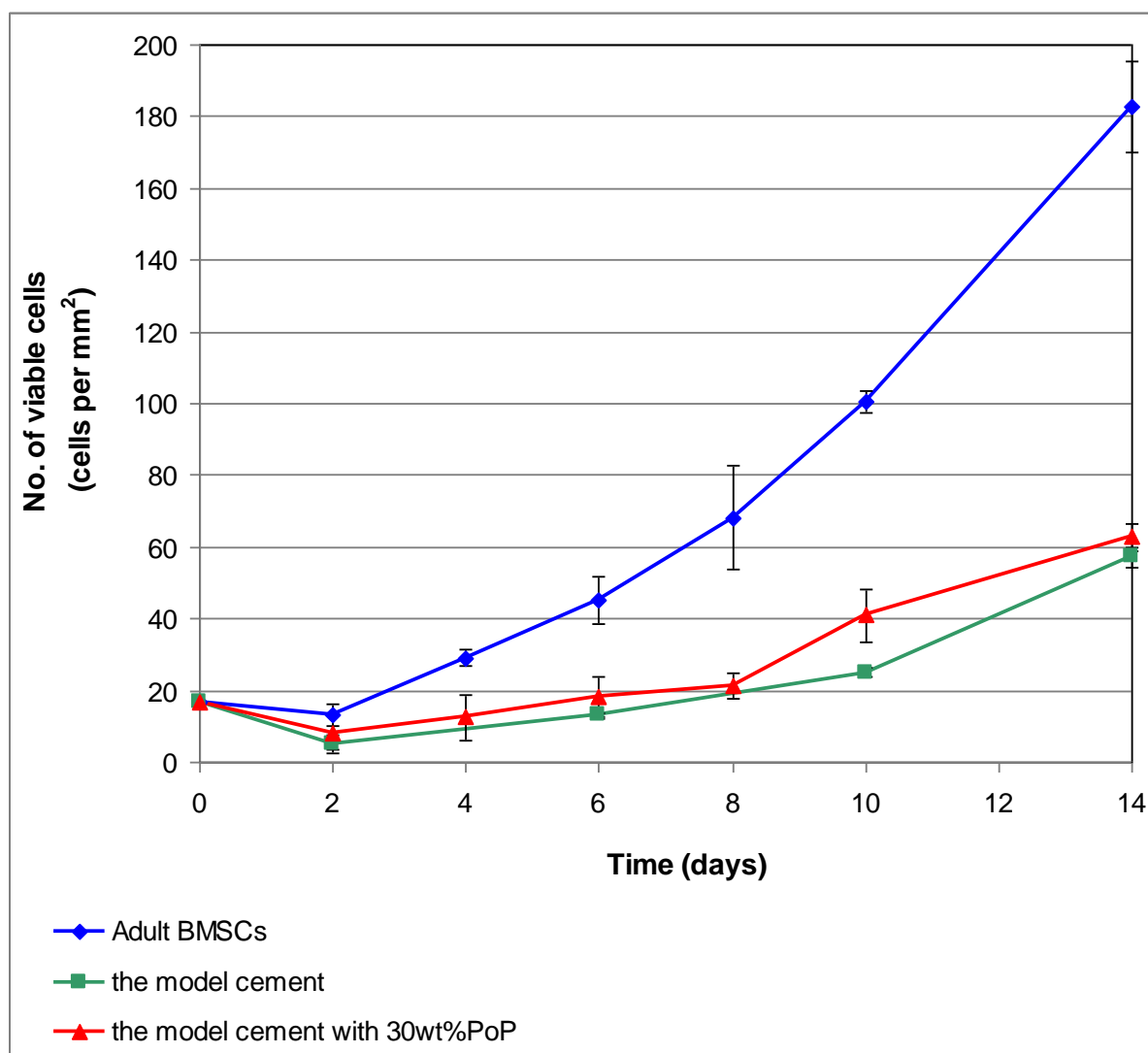


Figure 66: The density of viable adult BMSCs (seeded directly onto cements in total volume 4 ml of medium) in cultures that contain cements with or without PoP additions. Control cultures that consisted of adult BMSCs only had significantly higher cell densities compared with cultures with model cements containing 30 wt% PoP, which had slightly higher cell densities compared with model cement cultures. Error bars on the graph represent the standard deviations of the mean values plotted.

Cultures containing cements with or without 30 wt% PoP additions after 14 days had cell densities which were only a third of the cell density of control cultures which consisted of adult BMSCs only (see figure 66), which was significantly higher in comparison ($p < 0.05$).

RESULTS

Adult BMSCs attached to model cements with or without PoP additions and after 3 days in culture (see figures 67 and 68) but SEM examination showed adult BMSCs did not spread well on cement samples as shown in SEM photomicrographs (figures 67 and 68), which was unlike adult BMSCs from control cultures on glass cover slips where cells appeared to adhere closely and spread extending numerous filopodia (see SEM photomicrograph A of figure 67).

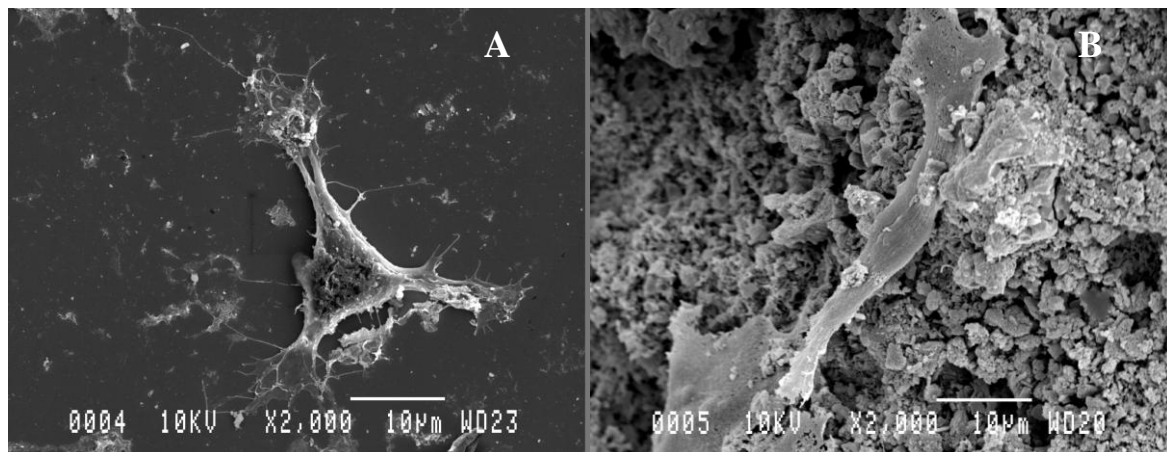


Figure 67: SEM photomicrographs of BMSCs in cultures on glass cover slips (A) and cultures with model cements without PoP additions (B). Cells appeared poorly attached to model cements since no filopodia were apparent when compared with BMSCs from cultures that consisted of these cells only after 3 days of culturing.

Cells appeared to be poorly attached to model cements containing 30 wt% PoP with few filopodia present, but cells were bridging over cement plates where pores were present in the cement structure (figure 68). However, no cells were observed to have attached to cements either with or without 10 wt% PoP when in culture for 14days (observed using SEM but no cells counted on cements).

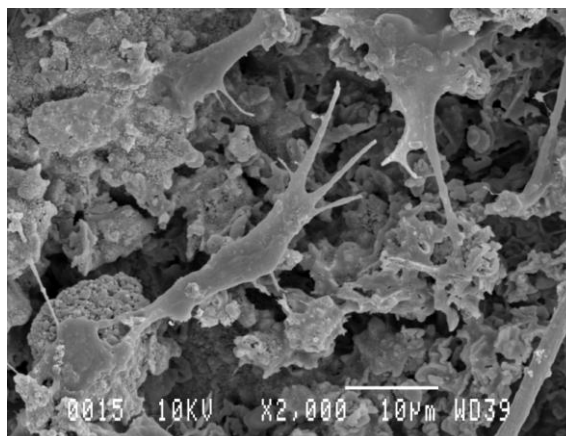


Figure 68: SEM photomicrograph of BMSCs in cultures containing model cements with 30 wt% PoP addition. Cells appeared poorly attached since few filopodia were present and appeared to bridge between cement particles where there are pores in the cement structure.

‘Live-dead’ staining of cultures indicated that some of the cells surrounding model cements had red nuclei, indicating that these adult BMSCs were not viable. In contrast, adult BMSC control cultures stained green indicating that cells were viable (figure 69, image A).

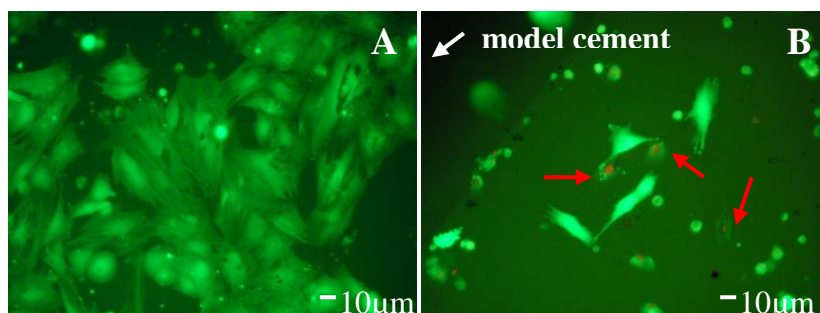


Figure 69: Phase contrast images of ‘live-dead’ stained cultures with (image B) or without model cements (image A). A few damaged adult BMSCs were observed to be attached to culture wells (indicated by red nuclei, see red arrow) near to model cements (dark black area in top left hand corner of image B) that did not contain additions, indicated by the red colour of cells.

7.4.2 Cells seeded into small volumes (50 μ l) of culture medium

Cultures containing cements had significantly fewer cells surrounding cements compared with the cell density of control cultures that consisted of adult BMSCs only ($p < 0.05$), as shown in figure 70.

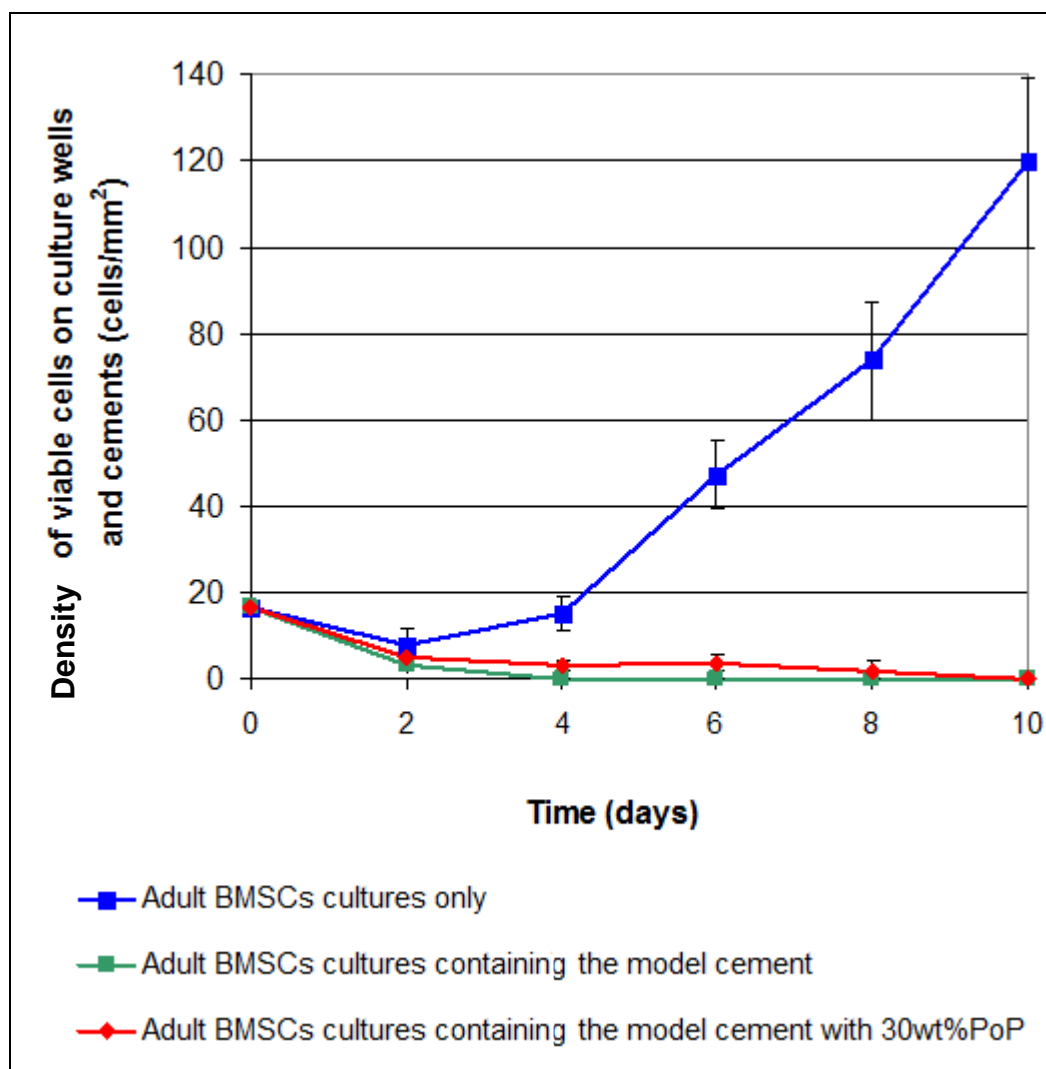


Figure 70: Total number of viable adult BMSCs (seeded directly onto cements in 50 μ l of culture medium and cultured for one hour) counted in cultures that contain cements with different compositions after being cultured for up to 10 days.

Cell proliferation increased continually after each time point for controls only (shown in figure 70), which was similar to adult BMSC control cultures which were seeded into a larger volume of

culture medium, see figure 66. Unlike adult BMSC cultures only, cultures containing model cements both with and without 30 wt% PoP had decreased cell densities which decreased with increased time in culture (see figure 70).

7.4.3 Cement samples placed onto established adult BMSC cultures

Cultures were exposed to cement after cell culture wells were covered with adult BMSCs. The addition of cements that contained 30 wt% PoP caused the density of cells attached to culture wells to decrease considerably compared with the control at 3 days (figure 71). There was cell proliferation observed in cultures containing model cements (with or without PoP) but less than the proliferation of control cultures.

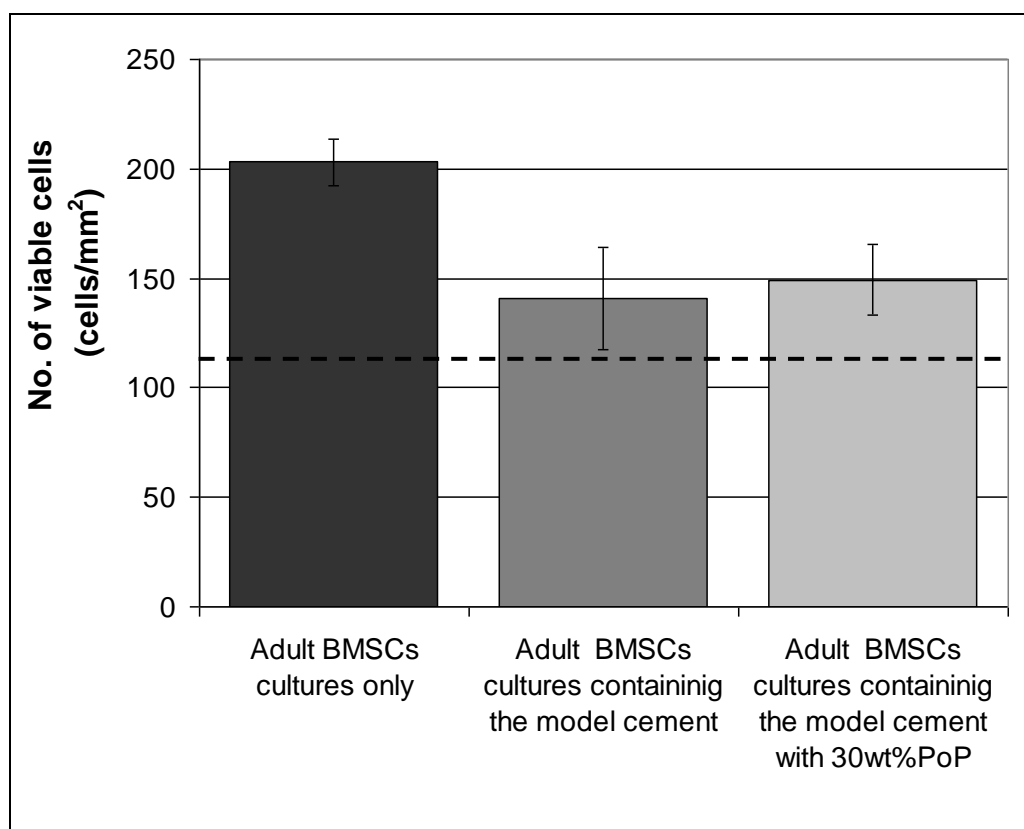


Figure 71: Density of viable neonatal BMSCs counted after 3 days in established cultures that contained the model cement with or without PoP additions cultured. The dashed line represents the cell density seeded into culture wells at the beginning of the experiment. Cell densities increased for all cultures but were higher for control cultures that consisted of adult BMSCs only compared with cultures containing cements.

RESULTS

Cell viability decreased slightly to 97 % for cultures containing model cements and 30 wt% PoP-containing cements by the end of the experimental period of 3 days but only decreased to 99 % for adult BMSC cultures without cement additions.

Cells appeared to be rounding up/ detaching (white rounded cells in figure 72) from culture wells when in close proximity to model cements with or without PoP, unlike cultures that consisted of adult BMSCs only. The density of cells surrounding cements with or without PoP additions appeared to be decreased with increased culture time, which left a zone of inhibition (an area surrounding the cement whereby no cells would remain attached or migrate towards) (see figure 72). The zone of cell growth inhibition appeared greater for non-modified model cements only when compared with model cements containing 30 wt% PoP.

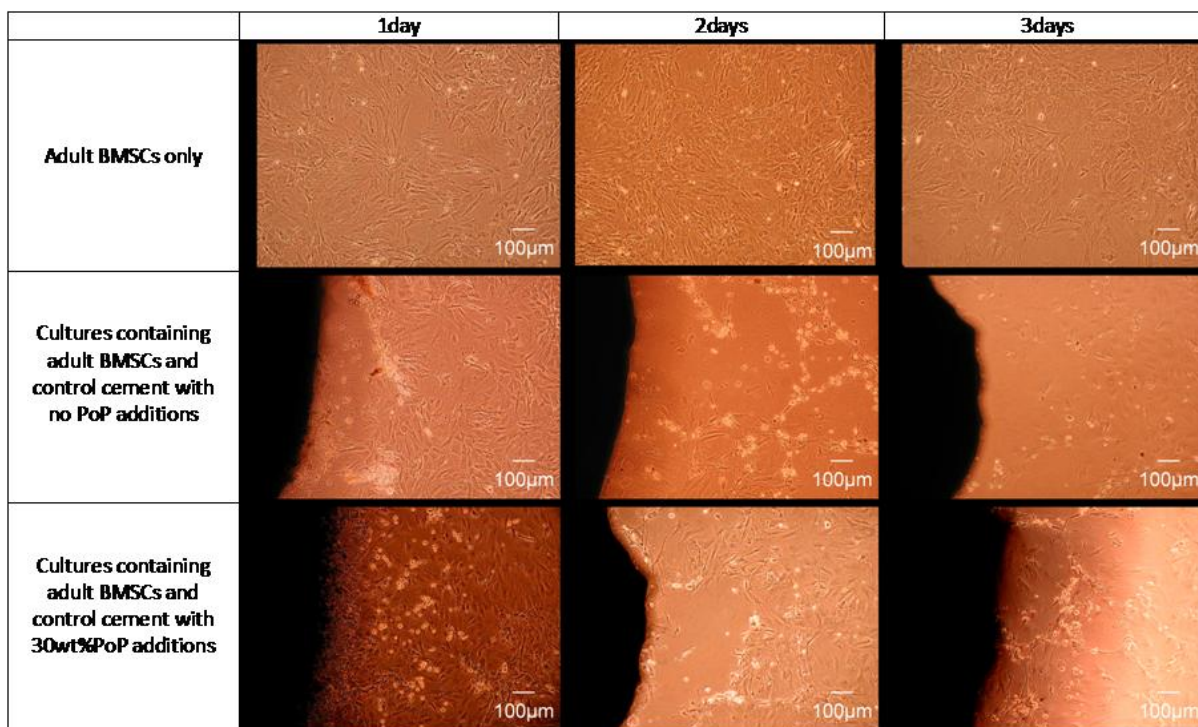


Figure 72: Phase contrast images of cultures with or without cement samples to show the density of cells in these cultures with time (3 days). Cell detachment was observed for cultures containing cements, which increased with time of culturing. Cells in control cultures appeared to proliferate with increased culture time since more cells were observed at 1 day compared with 3 days.

7.4.4 Cell attachment to cement samples only

Cultures set up in 96-well plates without cements had the greatest number of viable cells attached to culture wells as shown in figure 73 and there were no damaged cells counted indicating that the cell viability of these cultures was 100%.

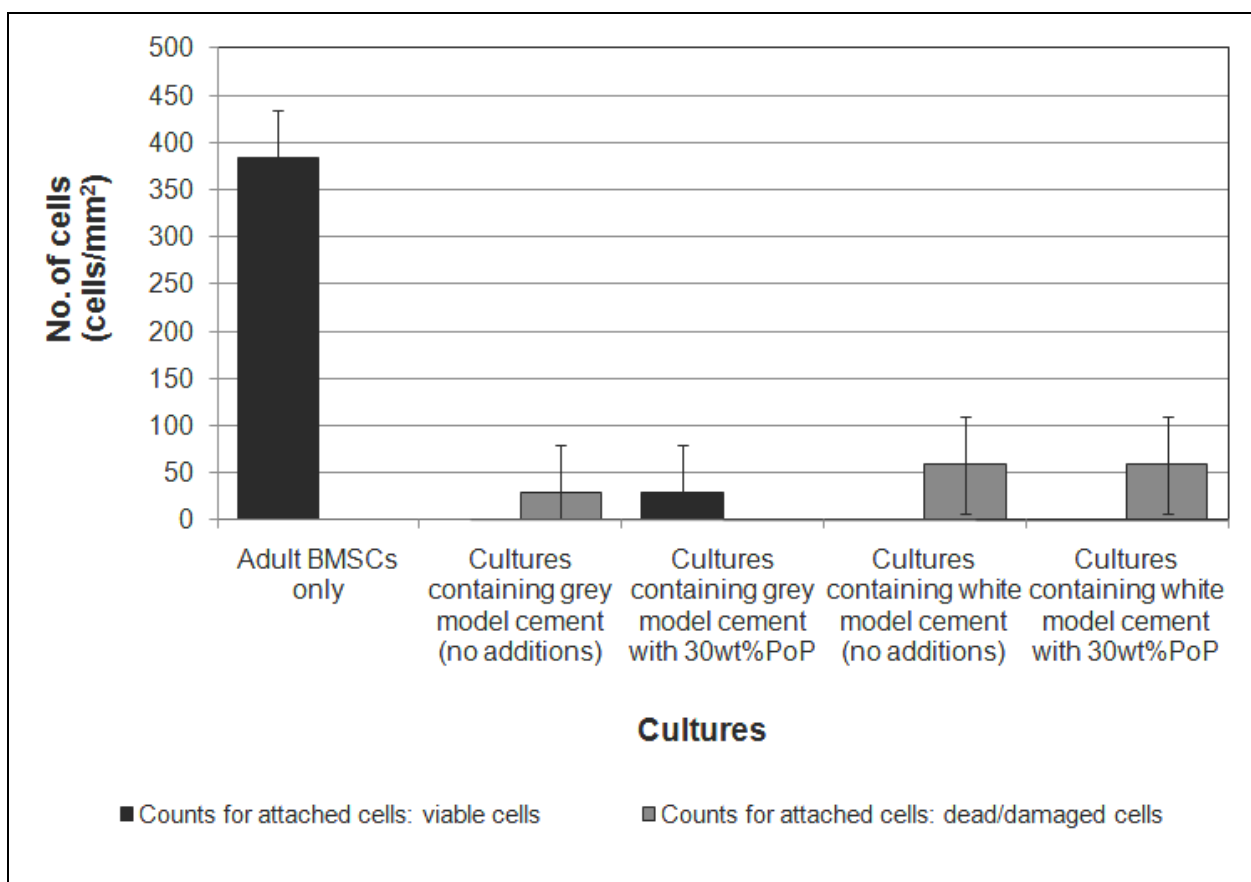


Figure 73: Cell attachment to cements and culture wells only after 3 days. Counts for the number of viable and non-viable cells were made as shown in the figure. Very few cells attached to grey model cement containing 30 wt% PoP. Error bars represent the standard deviations of mean values.

Cultures that consisted of cements with or without 30 wt% PoP as the substrate for cell attachment had significantly fewer cells that attached to cements ($p < 0.05$). Grey model cement containing 30 wt% PoP was the only cement that showed adherent, viable cells.

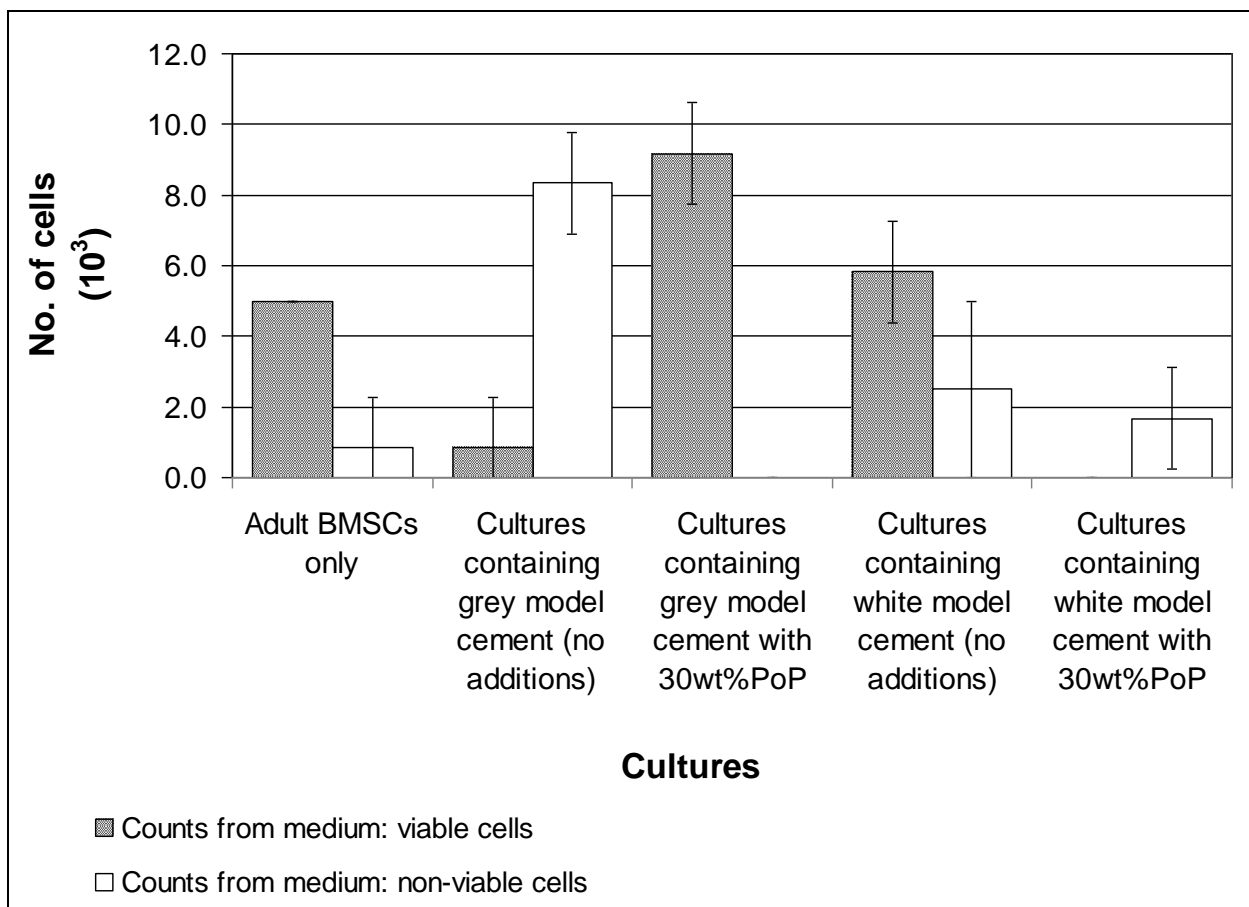


Figure 74: Bar chart to represent the number of viable and non-viable dead/damage cells in the medium of cultures containing cements with or without PoP after 3 days. Error bars represent the standard deviations of mean values. No viable cells were counted for cultures containing modified white model cements and no non-viable cells were present in culture medium from modified grey model cement-containing cultures.

A large number of viable adult BMSCs were counted in the medium of cultures containing grey model cement with 30 wt% PoP, however, there were fewer viable cells counted from the medium of cultures containing grey or white model cements without PoP additions (figure 74). There were three times as many non-viable cells counted in medium from cultures consisting of white model cements without additions of PoP as there had been in the medium from grey model cement with no PoP-cultures.

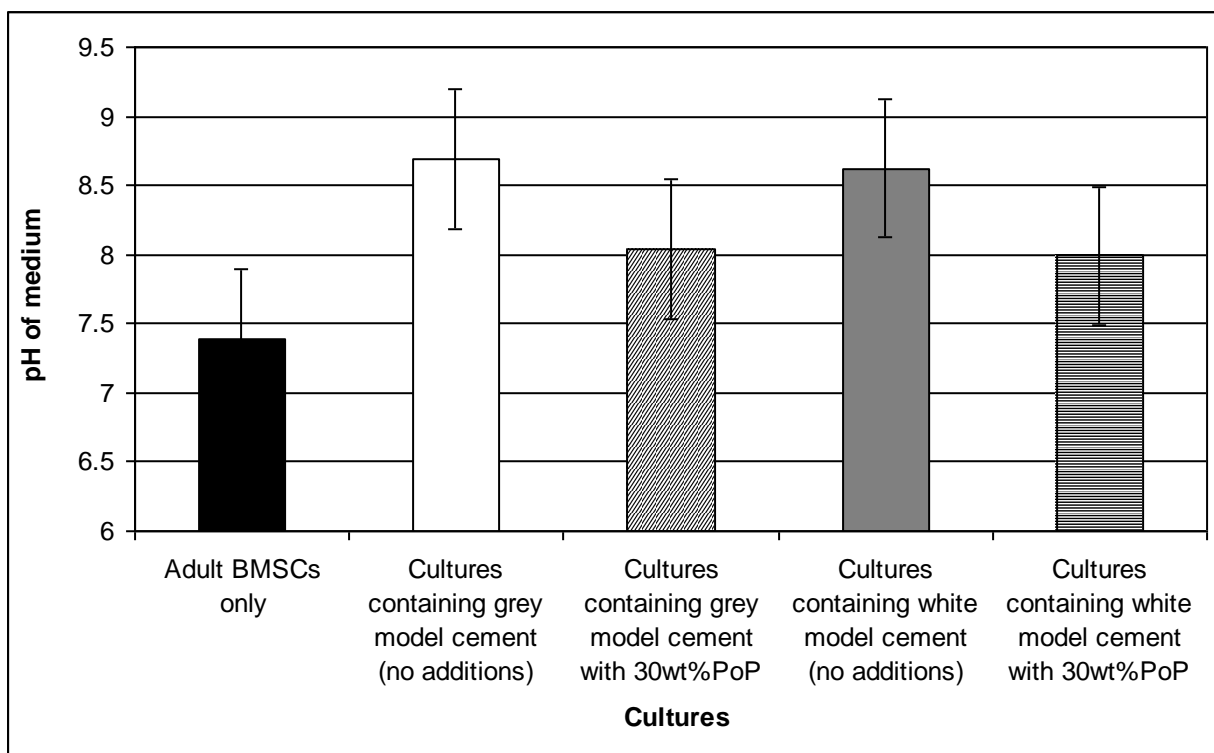


Figure 75: Bar chart to represent the pH of culture medium from cultures that contained the white or grey model cement with or without 30 wt% PoP additions after 1 day of culturing. The pH of culture medium increased with the presence of cements in cultures. Error bars represent the standard deviations of mean values.

The pH of culture medium from grey and white model cement containing cultures rose from pH7.4 (adult BMSC containing cultures only) to pH8.7 and 8.6 respectively (shown in figure 75) after 1 day, but the pH increased to a lesser extent for the culture medium removed from cultures containing grey and white model cements with 30 wt% PoP, only to pH8.0 as shown in figure 75. The original pH of culture medium before use was 7.3.

7.4.5 Cell attachment to cement paste only

Cell density decreased significantly for cultures containing 24 h set cement pastes with or without PoP (figure 76) when compared with the cell density seeded at the beginning of the experiment and cell viability decreased considerably (see appendix 4.1).

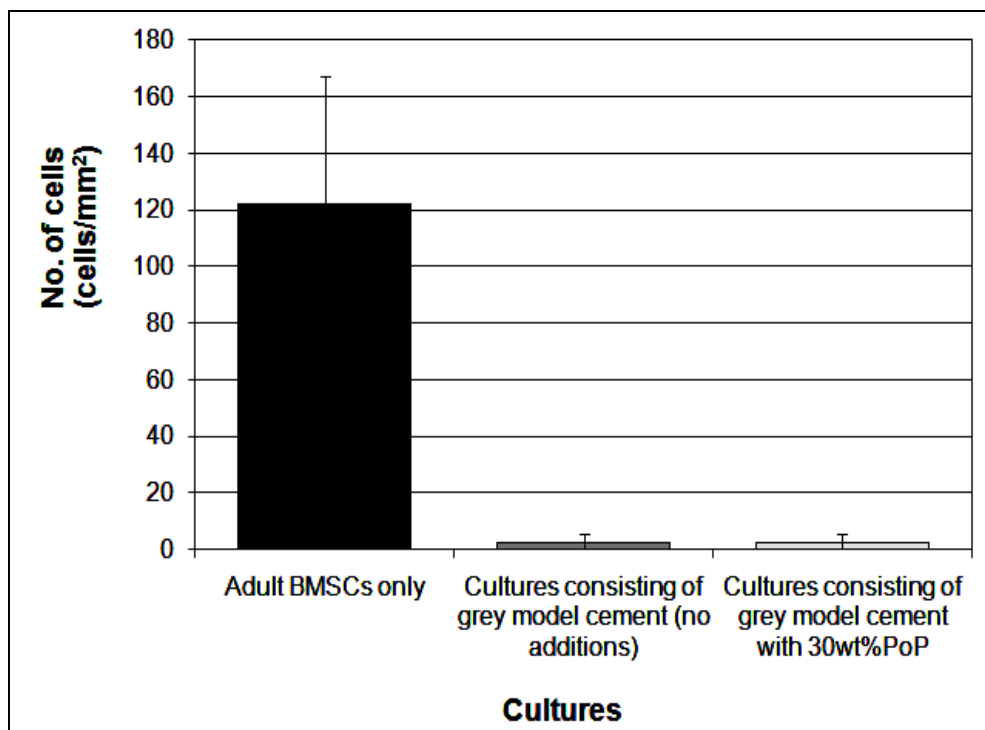


Figure 76: Bar chart to represent the density of cells attached to culture wells (for BMSC culture only) and cement pastes containing 30 wt% PoP. Error bars represent the standard deviations of mean values. Few cells attached to cement pastes compared with control cultures consisting of adult BMSCs only.

There were more BMSCs present in medium from cultures containing BMSCs only after 3 days when compared with cultures with cement pastes which can be seen in figure 77.

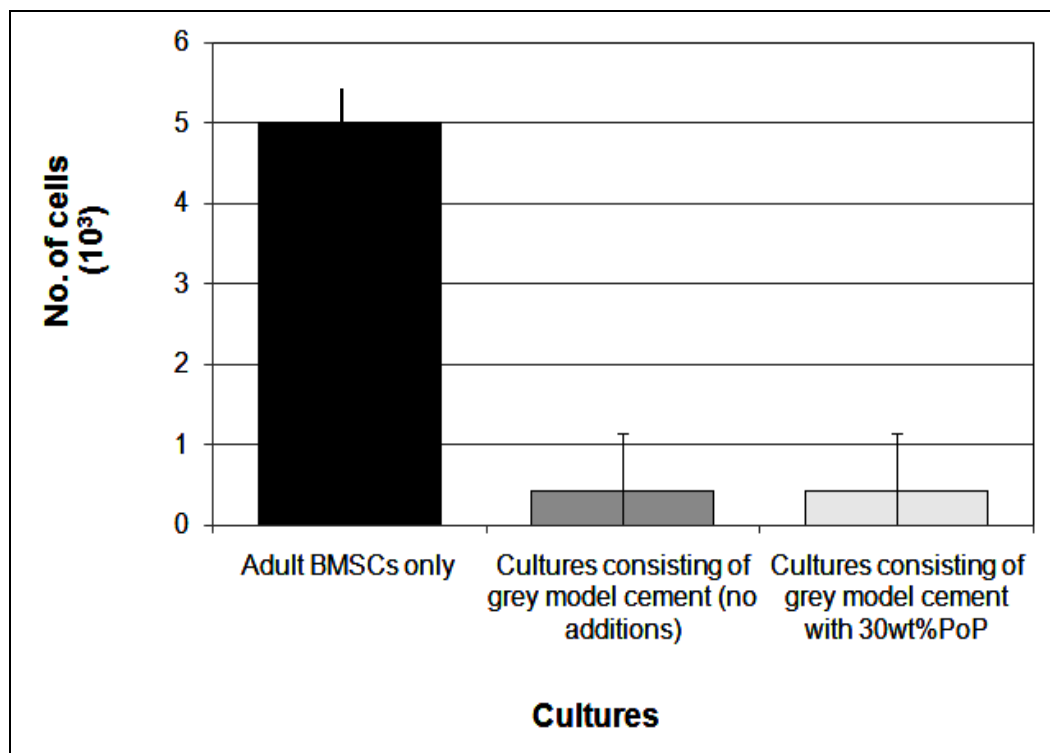


Figure 77: Bar chart to represent the total number of non-adherent, viable cells present in medium from cultures containing cement paste. Error bars represent the standard deviations of mean values. Few cells were present in the culture medium extracted from cultures that contained cement pastes compared with control cultures of adult BMSCs.

The pH of adult BMSCs cultures rose from 7.4 to 7.7 when cement paste containing 30 wt% PoP was within culture wells and increased to pH 8.0 for cultures containing the model cement paste without PoP additions after 1 day of culturing.

7.4.6 Cell cultures containing cements pre-washed in various solutions

The density of adult BMSCs attached to culture wells was significantly higher than in cultures that contained cements which had been washed in 70% ethanol. Cultures containing cements that were either washed or unwashed in distilled water, PBS or culture medium had similar densities for viable cells attached to culture wells as the control (figure 78). BMSCs that

RESULTS

were in cultures containing model cements washed with 70% ethanol were the least viable of all cultures (55 % viability) compared with other cultures (see figure 79).

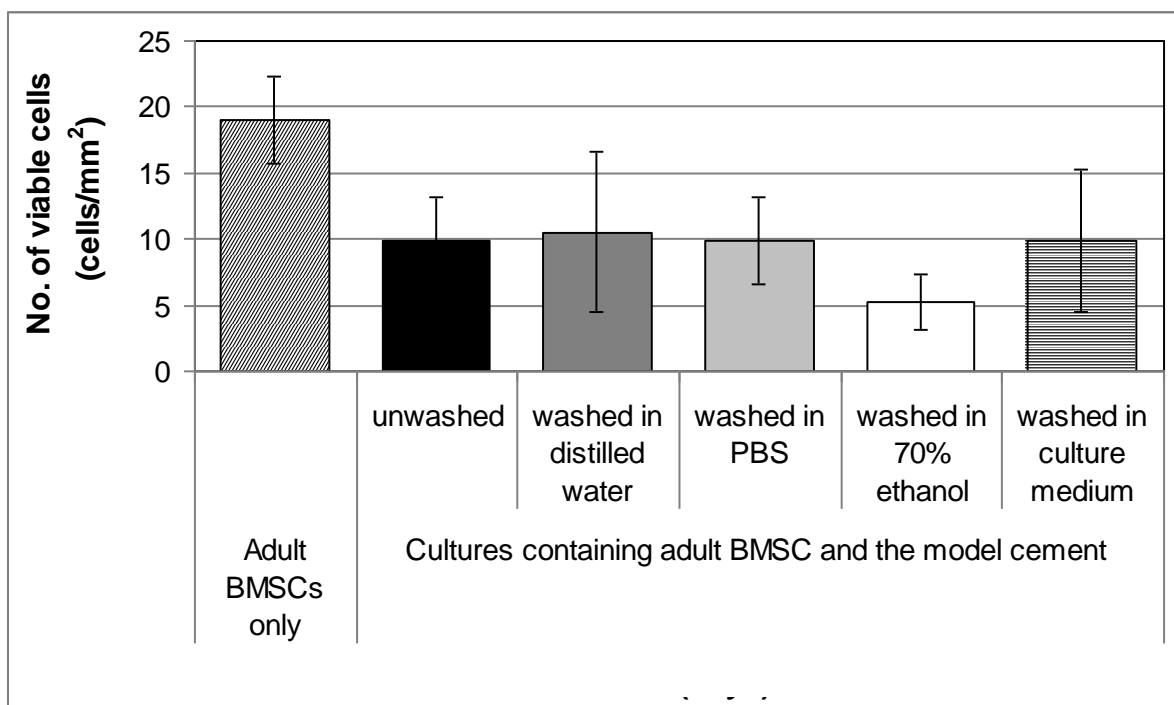


Figure 78: Bar chart to represent the density of viable adult BMSCs in cultures containing the model cement that were or were not washed with different solutions (cells attached to culture plastic). Cells only attached to cements that were washed in PBS. Error bars represent the standard deviations of mean values.

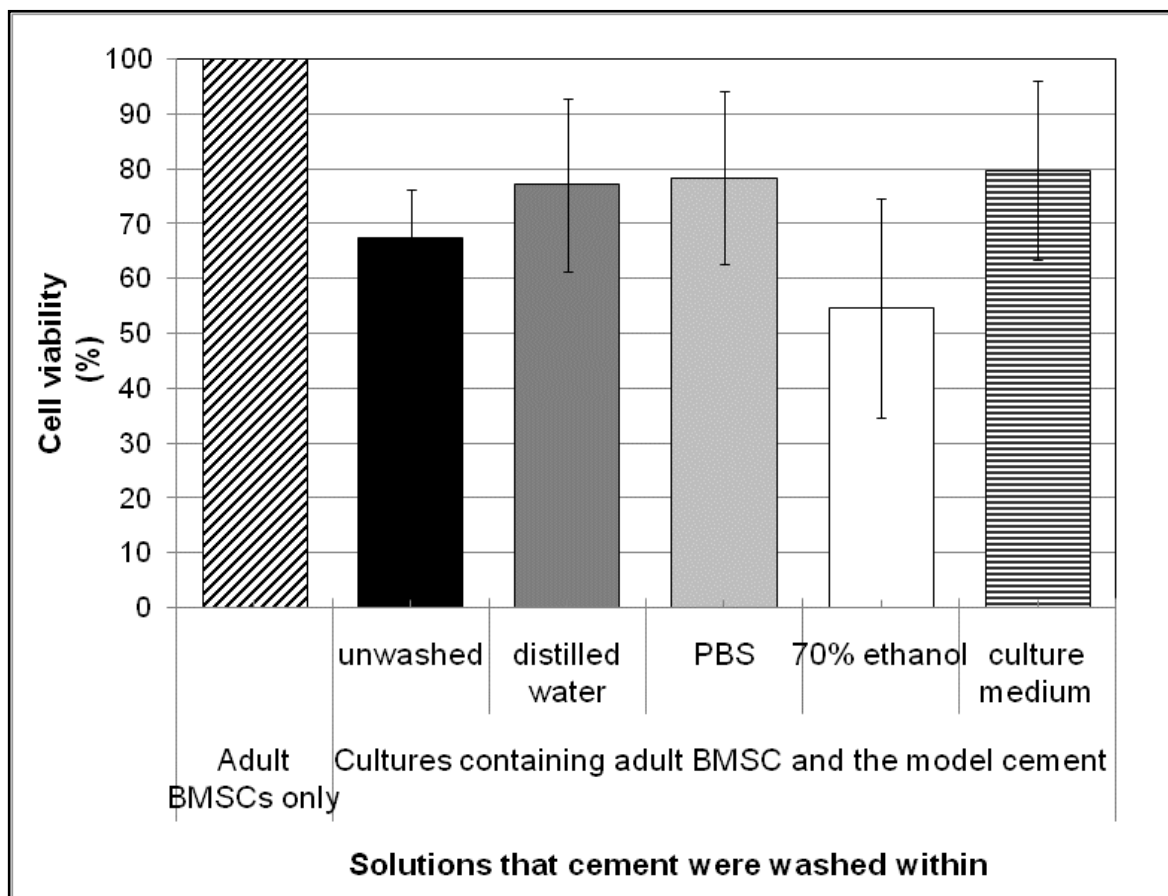


Figure 79: Bar chart to represent the viability of adult BMSCs attached to culture plastic in cultures that contained the model cement washed with different solutions compared with cells attached to culture wells of cultures containing unwashed cements. Error bars represent the standard deviations of mean values.

7.4.7 Cultures containing commercial MTA

Significantly fewer cells attached to culture wells (and cements) containing model cements compared with BMSC cultures only, as shown in figures 66 and 80 ($p < 0.05$) and fewer cells than cultures containing 10 wt% PoP-modified MTA or model cement and unmodified MTA.

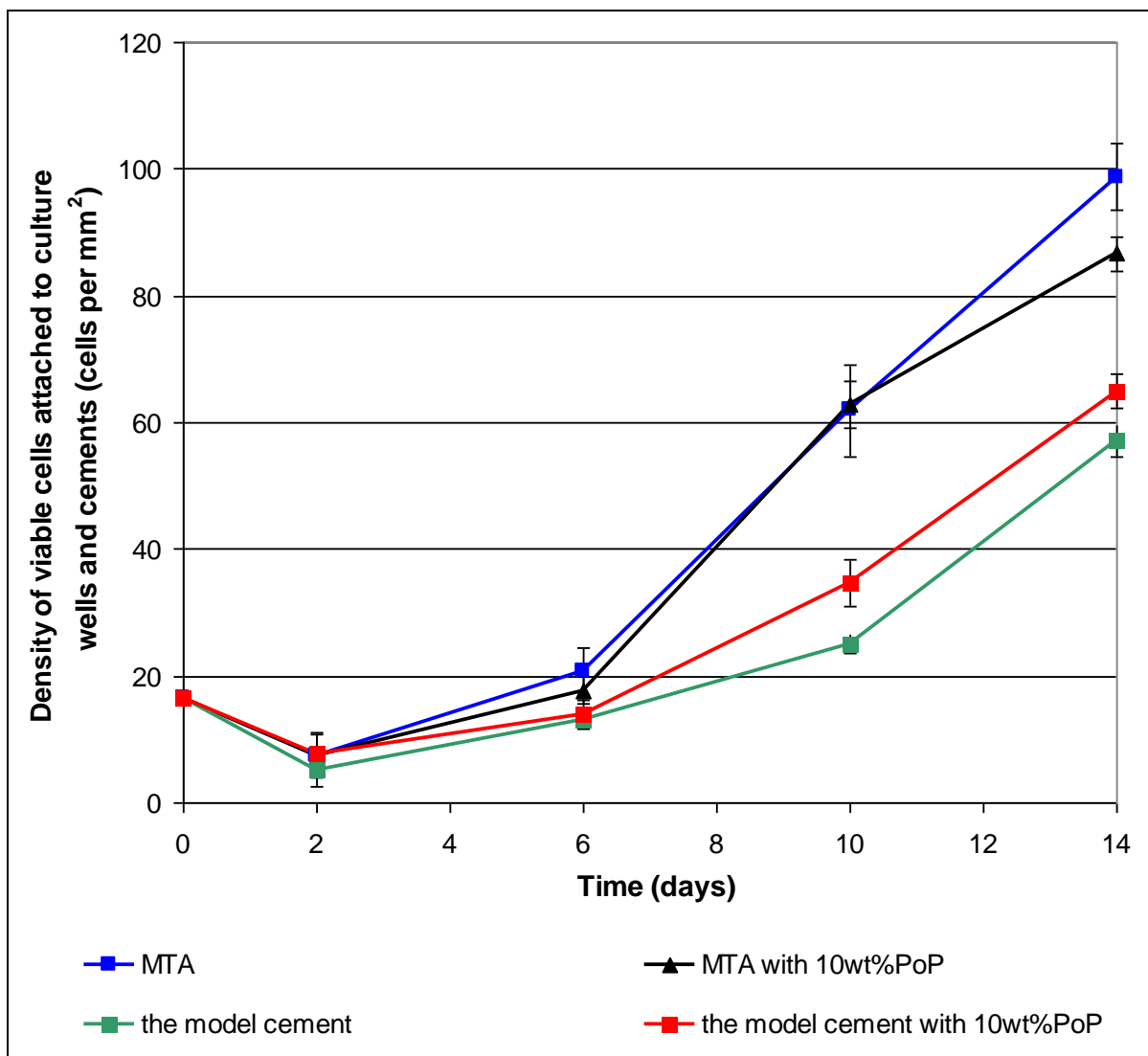


Figure 80: The density of viable BMSCs attached to culture plastic and cements in cultures containing MTA and the model cement with or without 10 wt% PoP. Error bars represent the standard deviations of mean values. Significantly higher cell densities were recorded for MTA-containing cultures at 14days compared with model cement-containing cultures.

Cultures containing MTA either with or without PoP showed significantly more viable BMSCs at 10 days ($p < 0.05$) compared with cultures that contained model cements with 0 or 10 wt% PoP (figure 80). MTA-containing cultures had significantly less BMSCs ($p < 0.05$) than control cultures at 10 and 14days of culturing. MTA-containing cultures had significantly more cells attached to culture wells when compared with model cement-BMSC cultures (figure 80). Cell density doubled for cultures containing model cements with or without PoP additions at

RESULTS

14days compared with 10 day cultures and was three quarters the density of 0 and 10 wt% PoP-MTA cultures at 14days (figure 80).

Inhibition zones for cell growth were observed in cultures containing cements (figures 81, images A, B, C and D). Cells did not attach to glass cover slips when in close proximity to MTA and model cements for both the PoP-modified and unmodified versions.

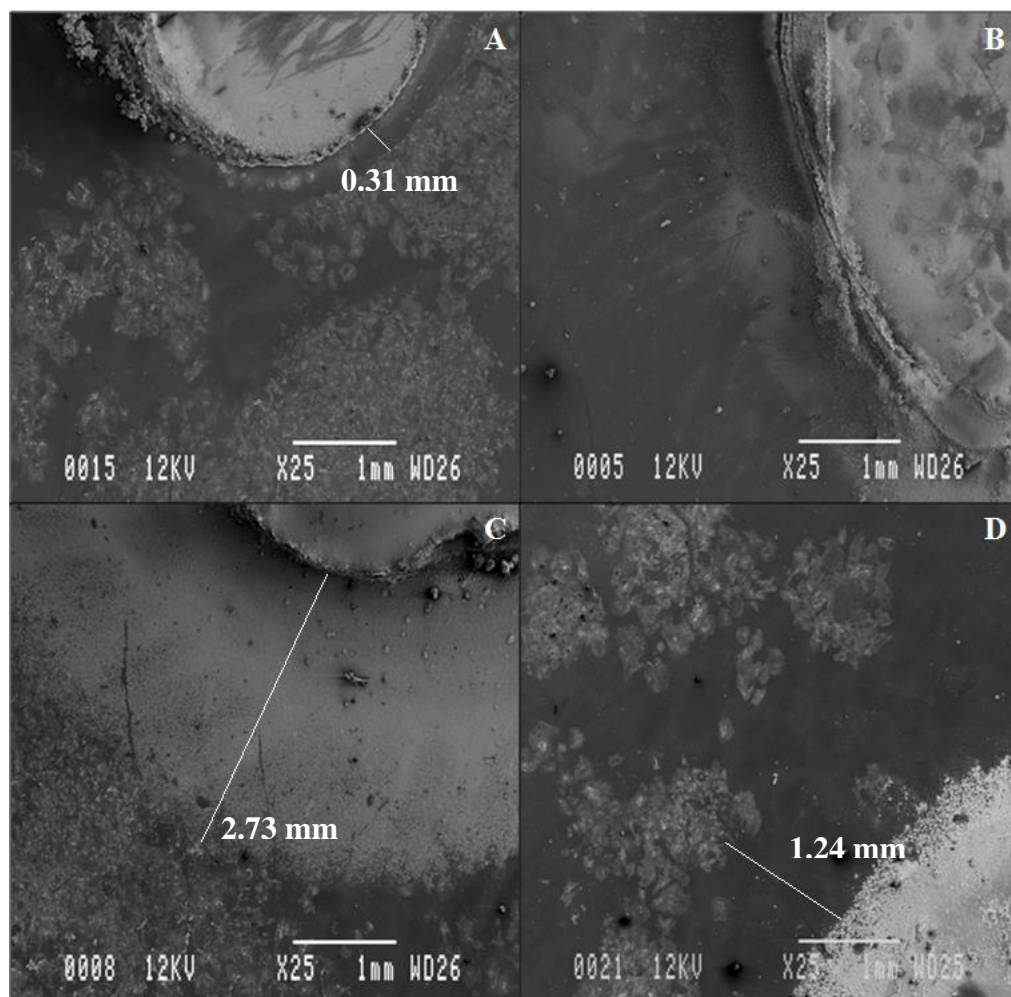


Figure 81: SEM photomicrographs of cells grown on glass slides in close proximity to cements. Images represent Adult BMSC cultures containing (A) MTA, (B) MTA modified with 10 wt% PoP, (C) the model cement and (D) the model cement containing 10 wt% PoP. Zones of cell growth inhibition were observed in cultures containing cements.

The inhibition zone for the growth and migration of adult BMSC was smaller around MTA (0.31 mm) compared with other cultures containing cements (>2.73 mm) as shown in figure 81. No

RESULTS

cells were observed in close proximity to MTA containing 10 wt% PoP-cultures which can be observed in image B of figure 81. The zone of cell growth inhibition surrounding model cements containing 10 wt% PoP was much smaller at 1.24 mm surrounding cement samples (figure 81, image D) when compared with MTA and the model cement without PoP (figure 81, images A, C and D). Unmodified model cements caused the inhibition zone of cell growth to double (2.73 mm) in size when compared with the effect of MTA and 10 wt% PoP-modified model cements in cultures (figure 81, images C and D). Cells in cultures containing MTA appeared to be fairly well attached to and spread on glass cover slips but did not attach as well as the control (see figures 82 and 83).

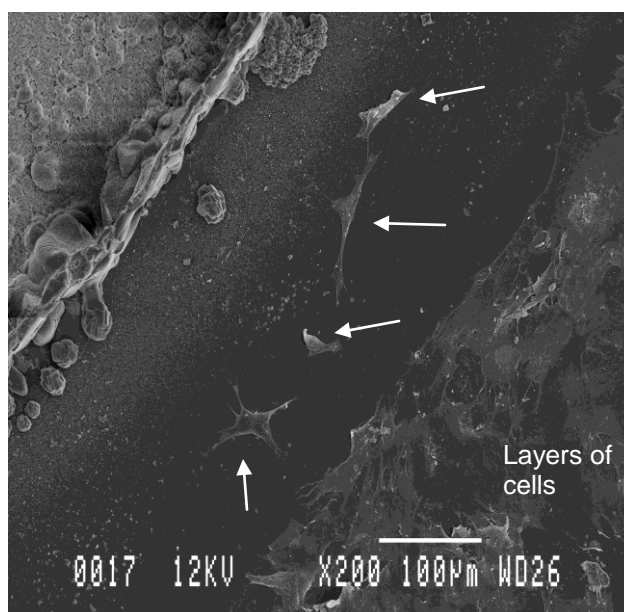


Figure 82: SEM photomicrographs of cells that had grown on glass slides in close proximity to MTA, indicated by white arrows and layers of cells labelled. A growth inhibition zone for BMSCs was observed surrounding MTA.

BMSCs attached to cultures glass in culture wells containing MTA without PoP additions appeared attached due to the flattened appearance of the cells and the presence of many filopodia but the cells nearest to MTA appeared less well-spread (figure 82). In comparison, BMSCs had

RESULTS

grown on glass cover slips that had no contact with cements in cultures were well spread (figure 83).

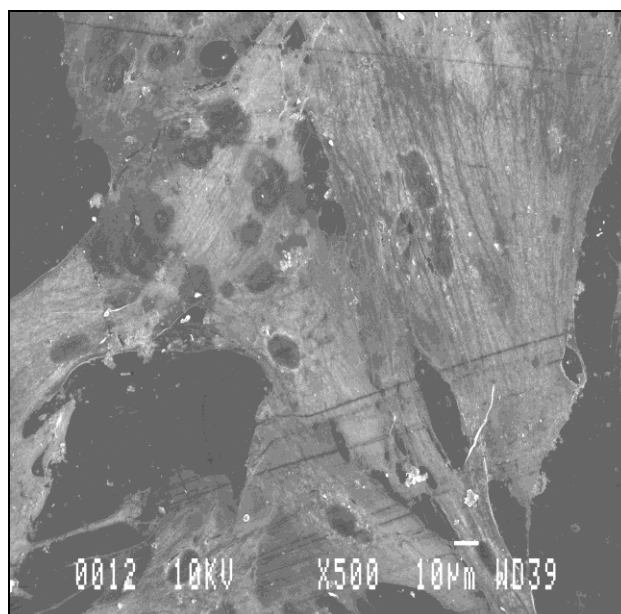


Figure 83: SEM photomicrographs of BMSCs that had grown on glass slides in culture wells. BMSCs were well attached and spread on the glass slides in culture wells, forming sheets of cells after 14days in cultures.

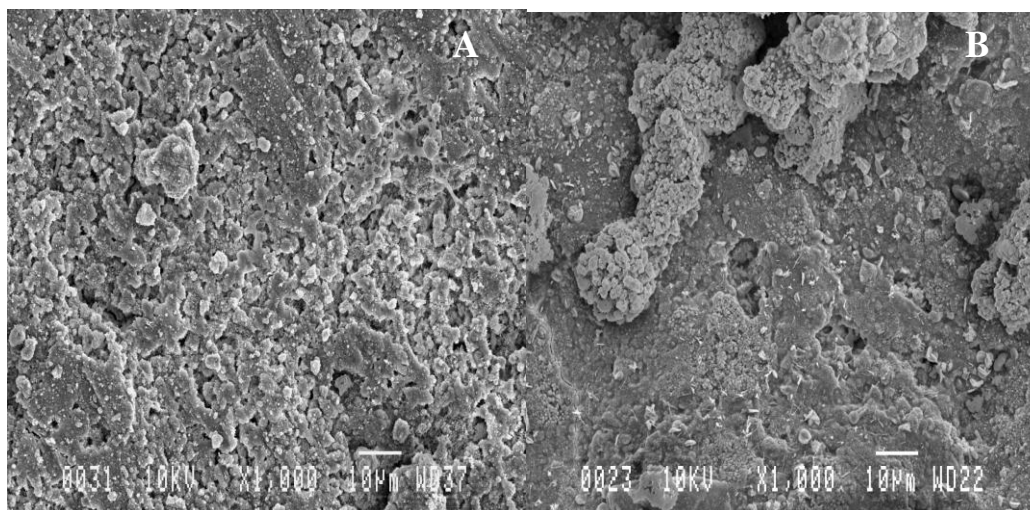


Figure 84: SEM photomicrographs of MTA (A) and MTA with 10 wt% PoP additions (B) that had been in cultures containing BMSCs for 14days. No BMSCs had colonised MTA or MTA with PoP.

RESULTS

No cells were observed to have colonised MTA and model cements with or without PoP when cultured for 14days (figure 84). In contrast, cells were located on model cements with or without PoP additions after 3 days in culture and were not well attached unlike the control (see section 7.4.1, figures 67 and 68). However, cells were not seen on cements with or without PoP after 14days in culture (figure 84). Some cell debris was apparent for model cements with PoP but not on model cements shown in figure 85, images A and B.

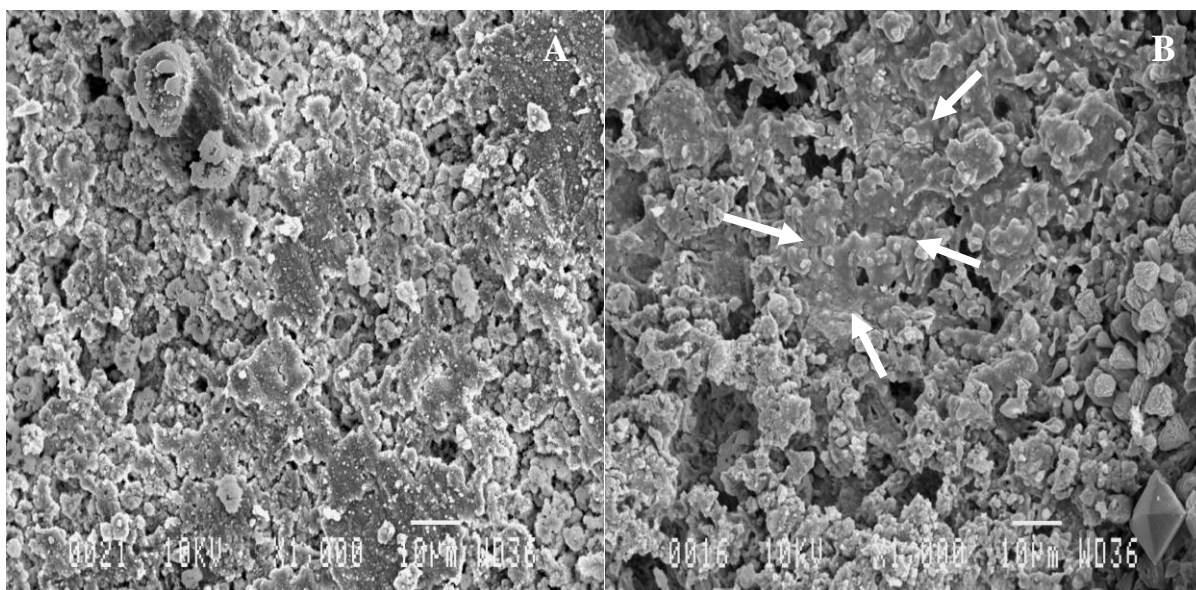


Figure 85: SEM photomicrographs of model cements without PoP (A) and 10 wt% PoP-containing model cements (B) that had been in cultures containing BMSCs for 14days. No BMSCs were found to have colonised on model cements with or without PoP and possible cell debris was observed on PoP-containing cements indicated by the arrows.

8 Sealing ability of model cements and MTA with additives

8.1 *Development of a tooth model: dye and ink permeation studies*

8.1.1 Measurements taken using a grid

Dye permeation was measured using a grid to make measurements less user dependent. The values recorded for Methylene blue dye permeation through cements were in a similar range to one another. Increased dye concentration had a greater impact on the depth of dye permeation into cement stored for 1 day in dye prior to 1 day of setting. Rhodamine B had the greatest depth of dye permeation as seen in figure 86.

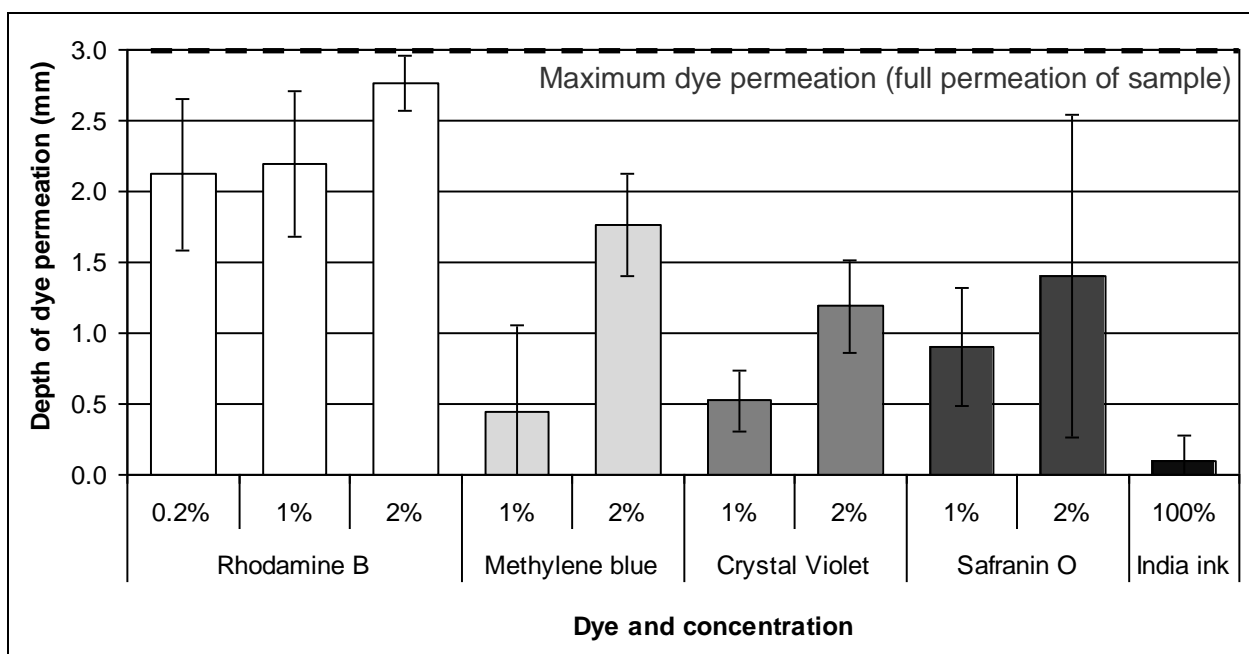


Figure 86: The permeation of various dyes at varying concentrations through model cements which were set for 1 days prior to being placed into dye for 1 days. Standard deviations were represented as error bars on the graph. 2 % Rhodamine B permeated through model cements the most when compared with other dyes at different concentrations.

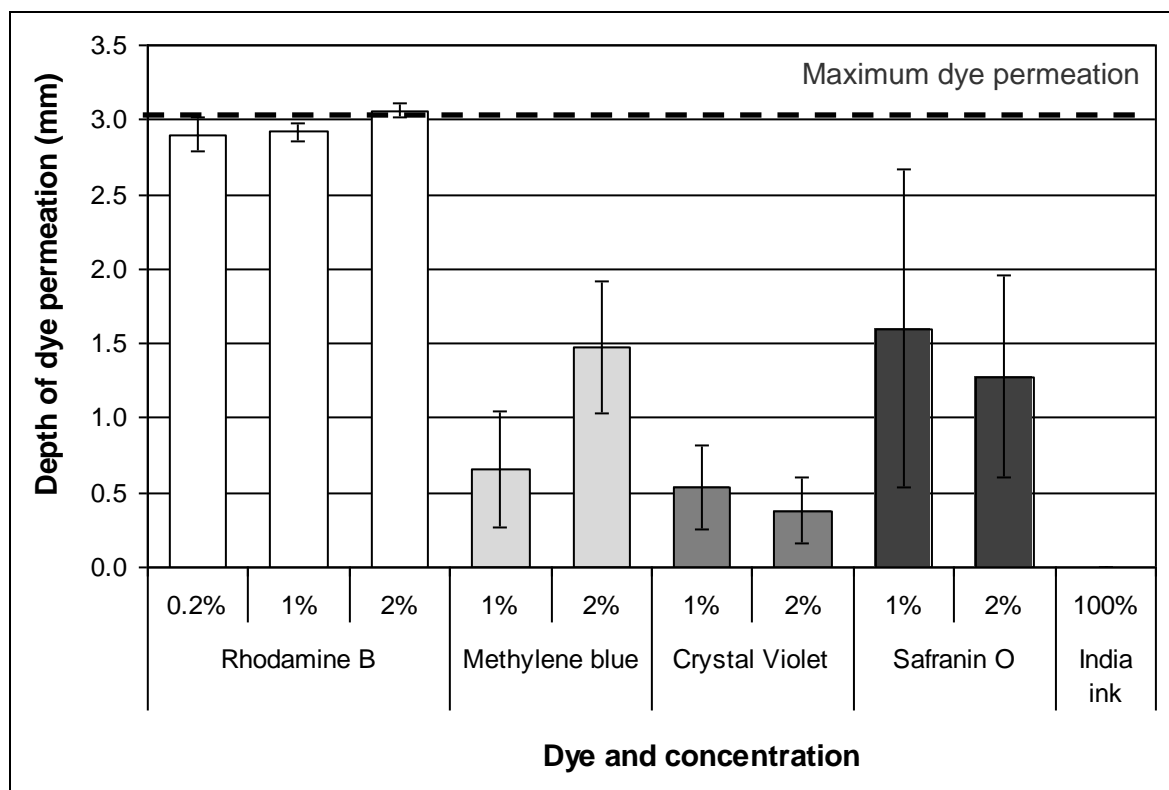


Figure 87: Bar chart to represent the permeation various dyes at varying concentrations through model cements which were set for 1 days prior to being placed into dye for 3 days. Standard deviations were represented as error bars on the graph. Regardless of the concentration, Rhodamine B permeated through model cements the most when compared with other dyes at different concentrations.

Cements stored in Rhodamine B dye were fully permeated after 3 days, which was not apparent after 1 day in this dye at any of the given concentrations of 0.2, 1 and 2 %. 1 % Methylene blue permeated cements less than 2 % Methylene blue dye, but this difference was not statistically significant (figure 87). Crystal violet dye permeated through cements after 3 days of storage in the dye similarly, regardless of concentration (figure 88). No India ink permeation was found when using a grid (figure 87) unlike the permeation of the ink into 1 day set cements stored in dye for 1 day (figure 86).

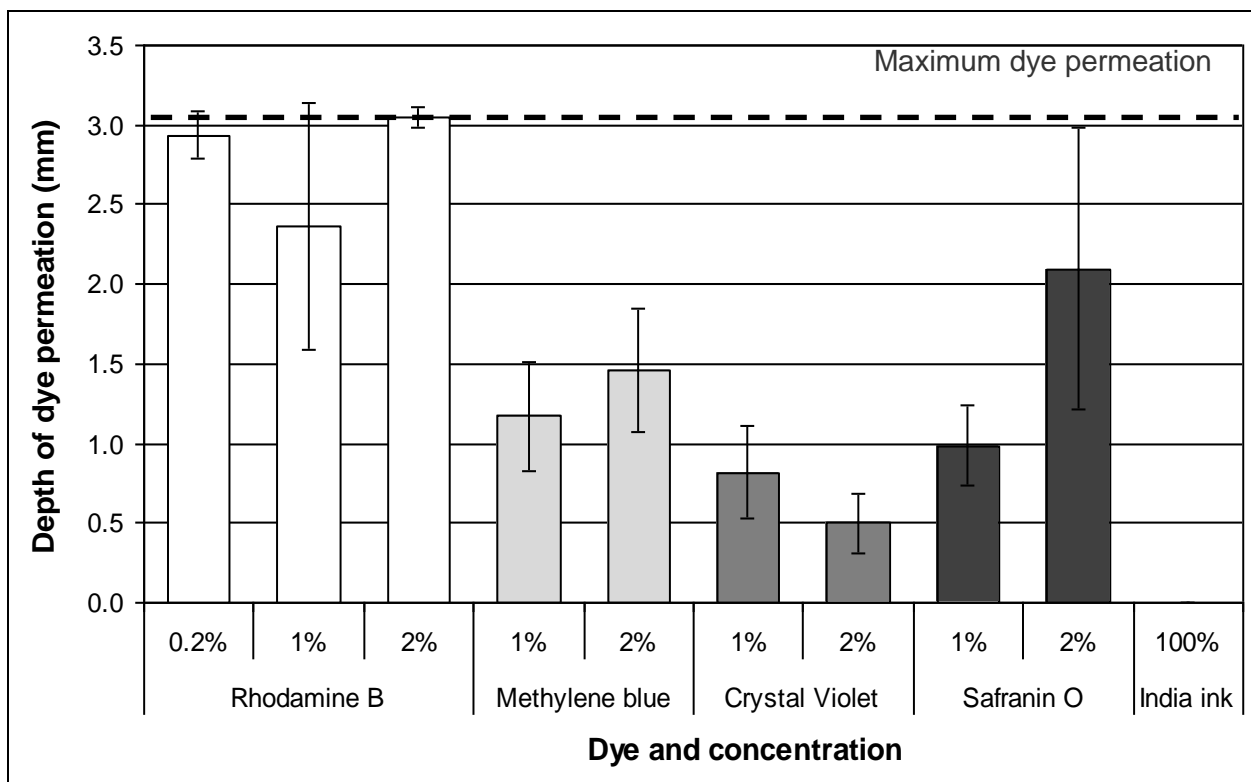


Figure 88: Bar chart to represent the permeation various dyes at varying concentrations through model cements which were set for 1 days prior to being placed into dye for 7 days. Standard deviations were represented as error bars on the graph. Rhodamine B permeated through model cements the most when compared with other dyes at different concentrations.

The same trends were present for dye permeation through model cements set for 1 day and stored in dye for 7 days (figures 86 and 88). 0.2, 1 and 2 % Rhodamine B dye fully permeated cements that were stored within it after 7 days (figures 88) when measured with a grid. 1 % Safranin O was significantly lower (1.0 ± 0.02 mm) than the dye permeation through model cements stored in 2 % Safranin O (2.1 ± 0.9 mm) was measured with a grid.

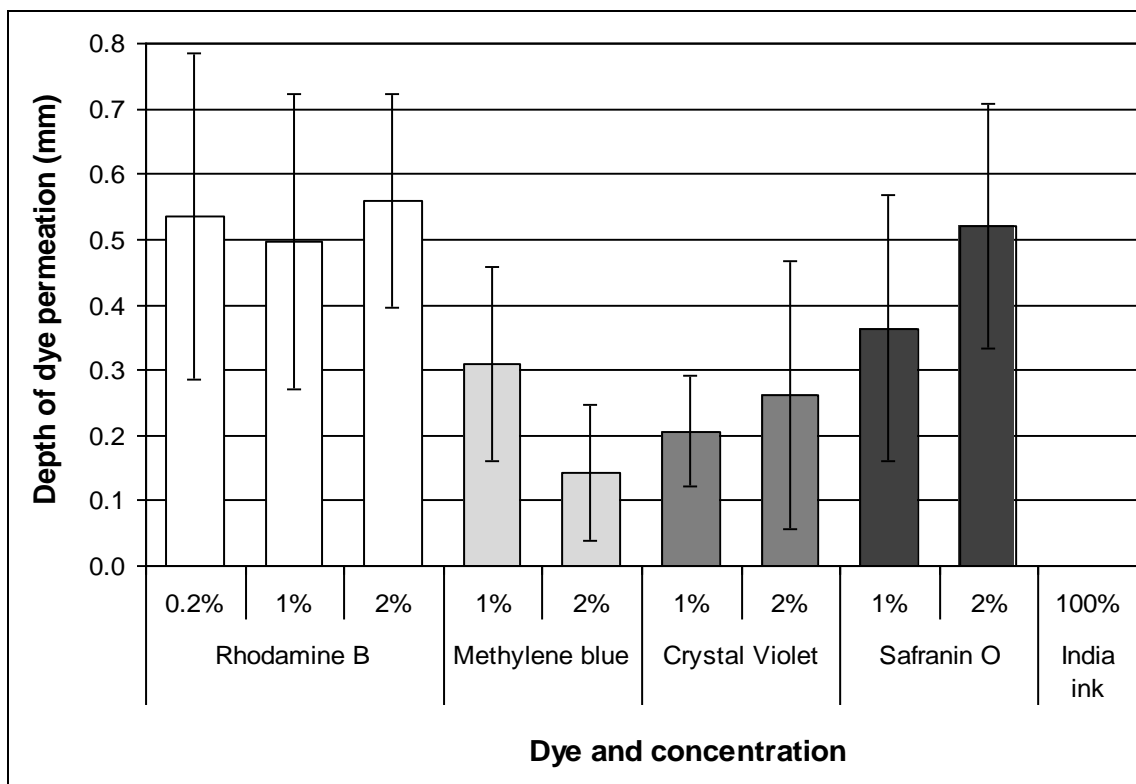


Figure 89: Depths of dye permeation through model cements with different dyes and concentrations which were stored for 3 days prior to being placed into dye solutions for 3 days. Standard deviations were represented as error bars on the graph. 2 % Methylene blue permeated through model cements the least when compared with other dyes at varying concentrations.

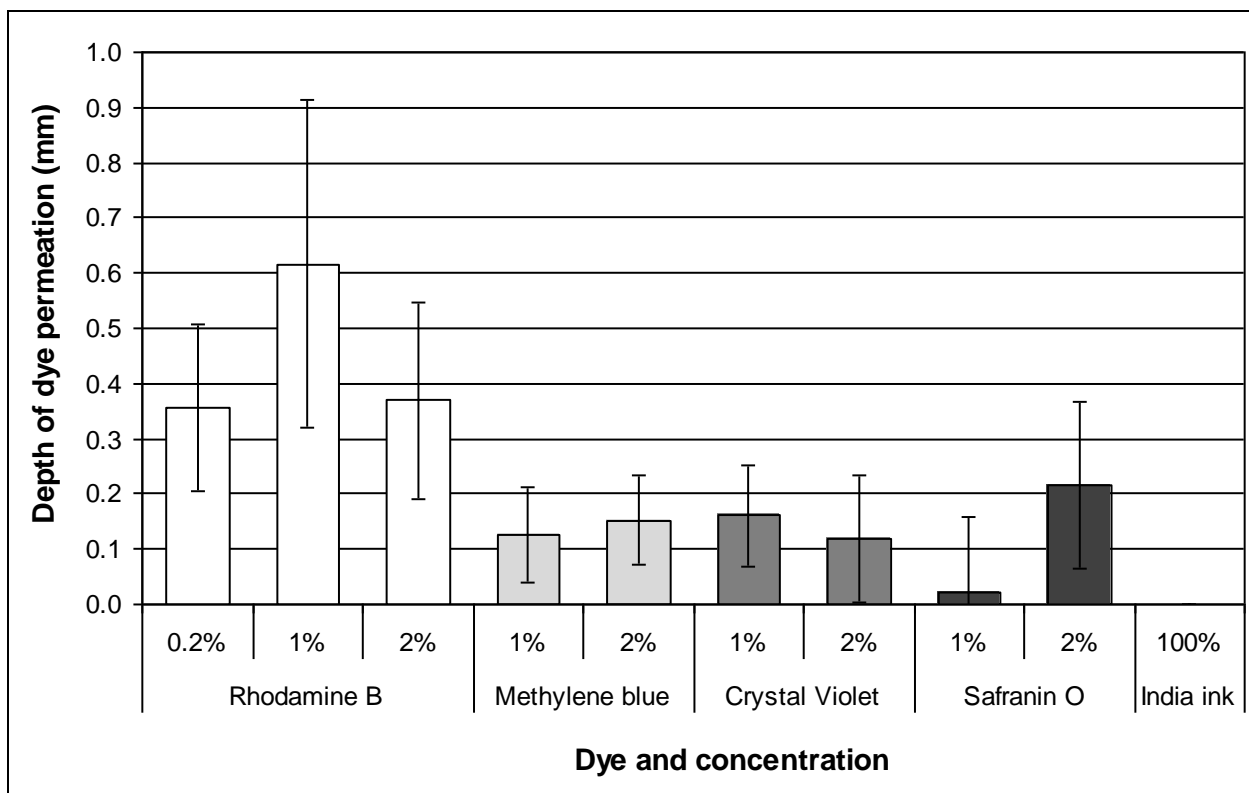


Figure 90: Bar chart to represent the depths of dye permeation through model cements which were stored for 7 days prior to being placed into different dyes and concentrations for 3 days. Standard deviations were represented as error bars on the graph. 1 % Safranin O permeated through model cements the least when compared with other dyes at varying concentrations.

The same trends were observed for the depths of dye permeation measured with the use of a grid measured for cements stored in dyes for 3 days prior to setting for 3 (figure 89) or 7 days (figure 90) and no significant differences ($p > 0.05$) were observed.

8.1.2 User dependency of the method established for determining dye permeation

To establish the effect of measurements made by different users of the method established for the measurement of dye and ink permeation, two users determined the dye permeation into cements (set for 1 day before dye immersion for 1 day) using the method using a grid established previously. The values recorded for dye permeation of different dyes with varying concentrations

RESULTS

into model cements differed for user 1 and 2 (figure 91). User1 measured that the dye permeation of cements stored in 0.2 % Rhodamine B and 2 % Safranin O solutions were significantly lower than the values recorded cements stored in these dyes by user 2 ($p < 0.05$).

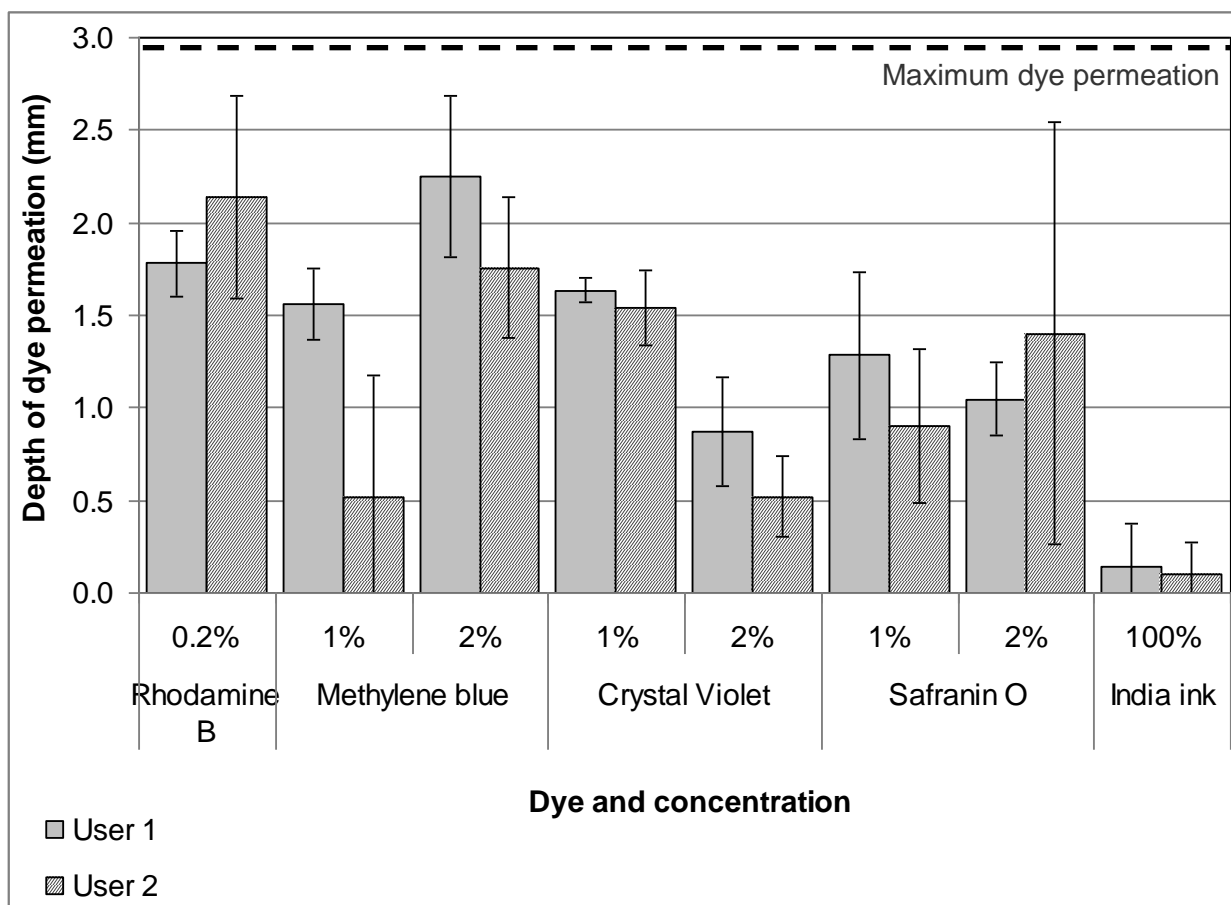


Figure 91: Bar chart to represent dye permeation through model cements, when different dyes and concentrations were used and the measurement of dye permeation was taken by two different users. Standard deviations were represented as error bars on the graph. Similar depths of 1 % Crystal violet were measured by the two users in the study.

The depth of dye permeation recorded by user 1 for cements stored in 1 % Methylene blue was two thirds higher than the value recorded by user 2. Values for permeation of dye into cements were statistically greater for cements with 2 % Crystal violet, 1 and 2 % Methylene blue and Safranin O permeation when measurements were taken by user 1 compared with the measurements for dye permeation taken by user 2. Measurements made for 1 % Crystal violet

permeation through cements measured by the two users were similar. India ink had the least dye permeation when measured by both users and these values for India ink permeation were the same as shown in figure 86.

Studies following those in this section were limited to the use of Crystal violet, Safranin O and India ink due to the interesting findings in this section (8.1).

8.2 *Effect of PLR on dye and ink permeation into cements*

8.2.1 **0.5 % Crystal violet permeation**

Cements with a PLR of 2.5 g/ml which had been set for 1 day before dye immersion had the greatest depths of Crystal violet permeation recorded. The values for dye permeation in cements with a PLR of 3.3 were less than one third the value of cements with a PLR of 2.5 g/ml under the same storage conditions (see appendix 1). Model cements with a PLR of 4.0 g/ml had the least dye permeation when compared with other cements that had been set for 1 day before dye immersion (figure 92).

After 10 and 30 days of setting before dye immersion, little dye permeation (~0.1 mm) was observed in cements with PLR 2.5, 3.3 and 4.0 g/ml unlike the amount of dye that was able to permeate through cements that had been set for just 1 day before dye immersion (figure 92) and significantly less ($p < 0.05$) than the amount of dye that had permeated into cements with a PLR of 2.5 g/ml after 1 day of setting (see figure 92).

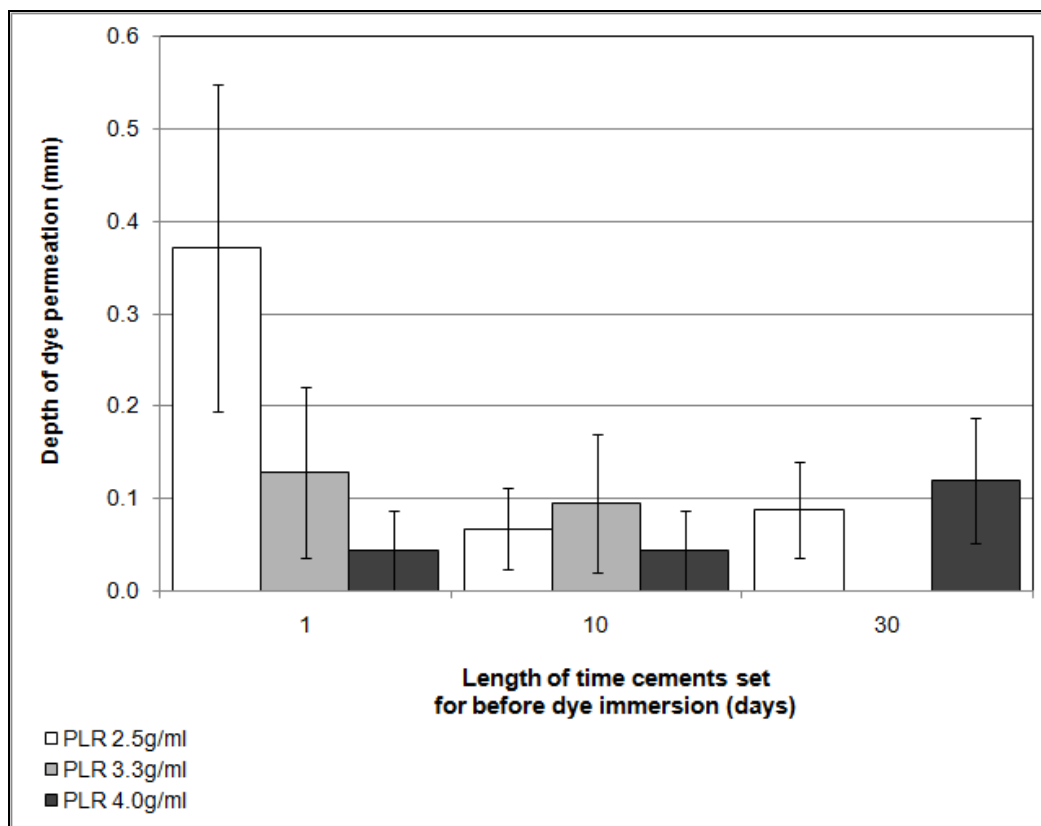


Figure 92: Depths of dye permeation (0.5 % Crystal violet) through model cements with different PLR which were stored for 1, 10 and 30 days prior to being placed into dye. Standard deviations were represented as error bars on the graph. Cements with a PLR of 2.5 g/ml had significantly more dye permeation after 1 days of setting compared with other PLR set for 10 and 30 days before dye immersion.

8.2.2.2 % Crystal violet permeation

Dye permeation decreased with increased setting time and increased PLR of the model cement when placed within 2 % Crystal violet (figure 93) but these trends were not so apparent when 0.5 % Crystal violet was used (figure 93). The greatest depth of dye permeation was recorded in cements with a PLR of 2.5 g/ml that were stored in dye after 1 day of setting (figure 93). Dye permeation decreased by one half of the value of dye permeation recorded for cements with a PLR of 2.5 g/ml stored for 1 day before dye immersion, when the PLR increased to 3.3 g/ml and further decreased when the PLR increased to 4.0 g/ml (figure 93).

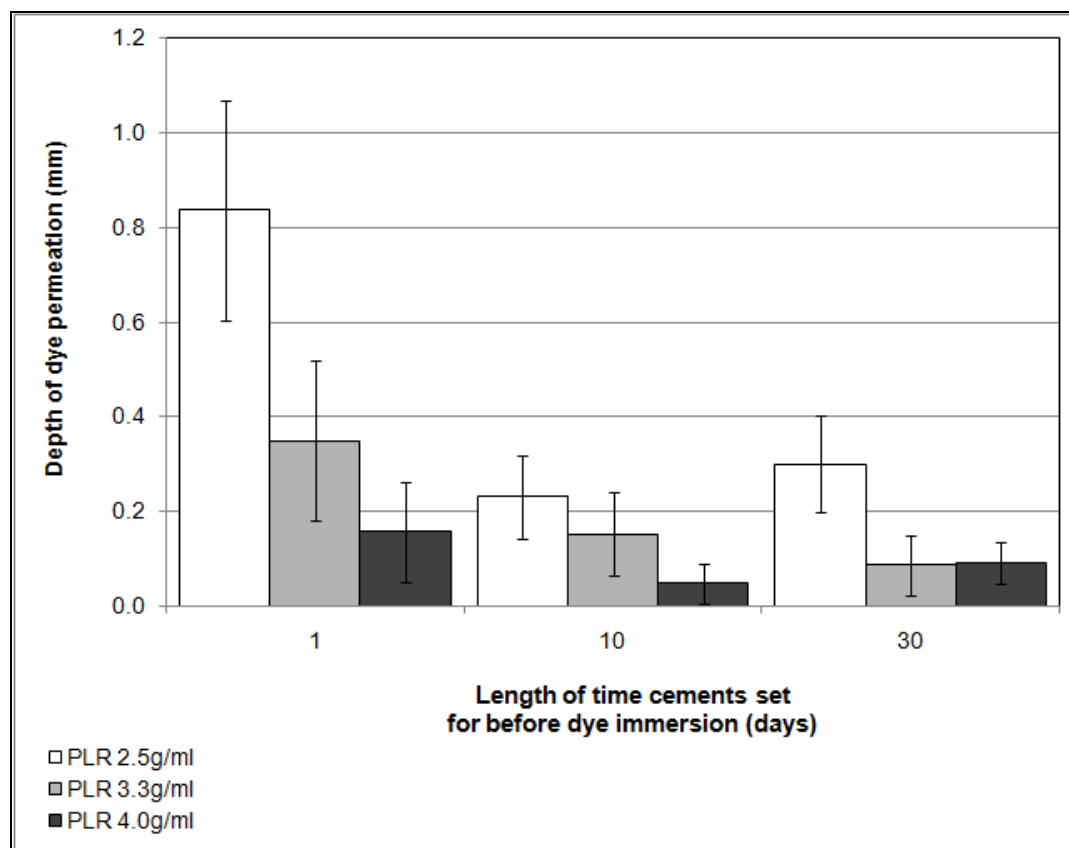


Figure 93: Depths of dye permeation through model cements with different PLR which were stored for 1, 10 and 30 days prior to being placed into 2 % Crystal violet solutions. Standard deviations were represented as error bars on the graph. Cements with a PLR of 2.5 g/ml had the highest values recorded for dye permeation when compared with other PLR stored for the same length of time before being placed into dye.

The values recorded for dye permeation into cements with a PLR of 2.5 and 3.3 g/ml that were stored for 1 days before 2 % Crystal violet immersion, decreased by over 50% when these cements had been stored for 10 days before dye immersion. After 30 days of setting, model cements with a PLR of 2.5 g/ml placed into dye had similar depths of dye permeation as cements set for 10 days with the same PLR. Cements with PLR 3.3 and 4.0 g/ml had little permeation of 2 % Crystal violet recorded after 30 days of storage before dye immersion (figure 93).

The concentration of Crystal violet was selected as 2 % for subsequent studies since this concentration provided better contrast with the cement samples when the dye had permeated cement samples.

8.2.3 0.5 % Safranin O permeation

Similar to the trend observed for permeation of 0.5 % Crystal violet into cements (figure 94), model cements with a PLR of 2.5 g/ml that had been stored for 1 day had the greatest amount of dye permeation compared with cements with PLR 3.3 and 4.0 g/ml. When model cements of all PLR were set for 10 and 30 days before 0.5 % Safranin O immersion, the dye permeation was reduced considerably to under 0.1 mm as shown in figure 94.

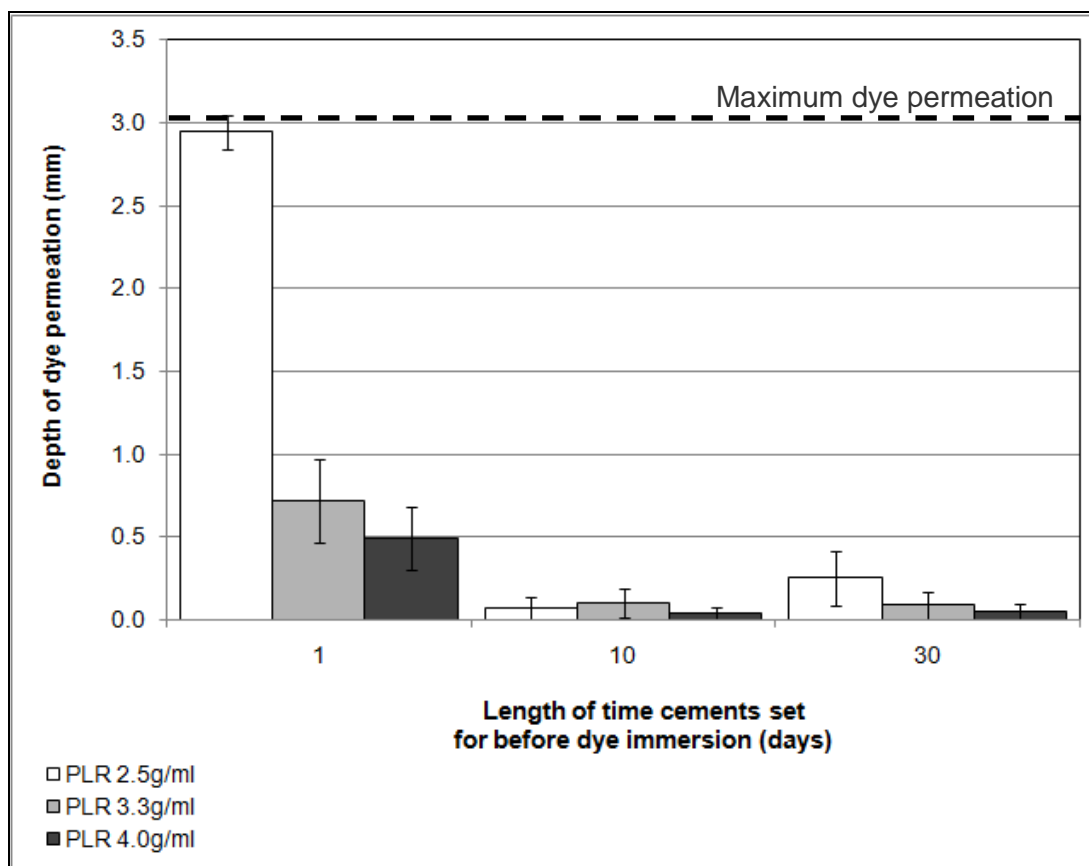


Figure 94: The depth of Safranin O (0.5 %) permeation through model cements with different PLR. Standard deviations were represented as error bars on the graph. More 0.5 % Safranin O permeated through model cement with a PLR of 2.5 g/ml which had been stored for 1 day prior to immersion in dye.

8.2.4 2 % Safranin O permeation

Increased setting time of cements and increased PLR caused the dye permeation to decrease considerably (see figure 95). 2 % Safranin O fully permeated model cements with a PLR of 2.5 g/ml that had been set for 1 and 10 days before dye, however, when cements with a PLR of 2.5 g/ml were set for 30 days prior to dye immersion, dye permeated into cements decreased significantly to 0.12 ± 0.05 mm ($p < 0.05$), shown in figure 95.

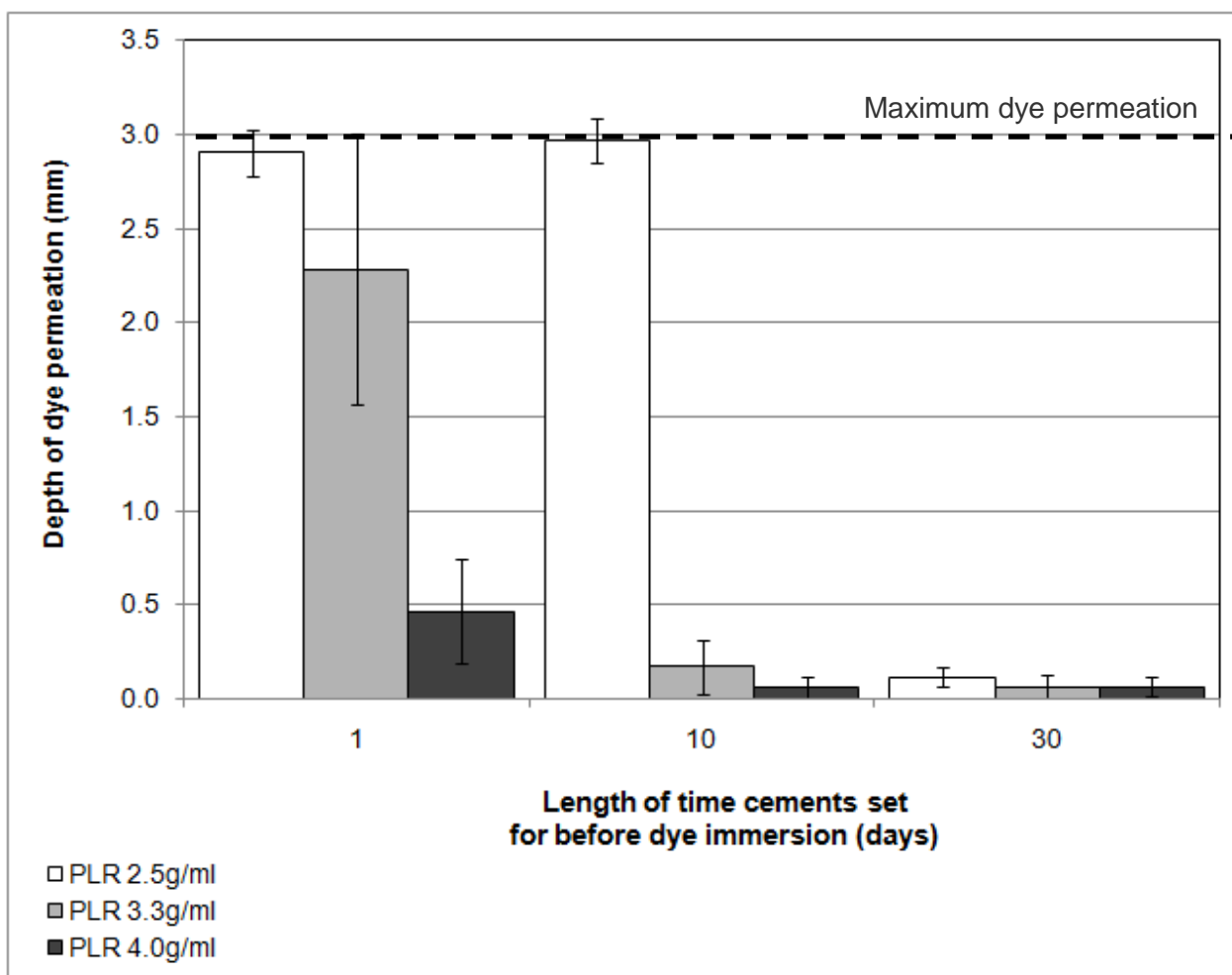


Figure 95: Bar chart to represent the depth of Safranin O (2 %) permeation through model cements with different PLR. Standard deviations were represented as error bars on the graph. Model cement with a PLR of 2.5 g/ml had the greatest depth of dye permeation when stored for 1 and 10 days prior to immersion in 2 % Safranin O.

Cements with PLR 3.3 and 4.0 g/ml that were set for 10 days prior to dye immersion had the least dye permeate into cements compared with model cements with a PLR of 2.5 g/ml set for the same length of time before immersion in 2 % Safranin O. The depth of dye permeation remained low (<0.5 mm) when cements with PLR 2.5, 3.3 and 4.0 g/ml were set for 30 days before 2 % Safranin O immersion (figure 95).

The concentration of Safranin O was selected as 2 % for studies that were to follow since this concentration provided better contrast with the cement samples when the dye had permeated cement samples.

8.2.5 India ink permeation

There was little permeation (<0.05 mm) recorded for cements at varying PLR which had been stored for different lengths of time before India ink immersion (see appendix 5.1 for values).

8.3 *Effect of PoP addition on dye and ink permeation through cements*

8.3.1 Crystal violet permeation

Increased PoP content to cement increased the depth of permeation significantly ($p<0.05$) but was only apparent when cements were set for 1 day prior to immersion in dye. Crystal violet permeation through cements with PoP that had been stored in dye for 3 days, prior to 1 day of setting was significantly greater ($p<0.05$) than cements that did or did not contain PoP and the permeation of Crystal violet decreased considerably with increased setting of cements (10 and 30 days), shown in figure 96.

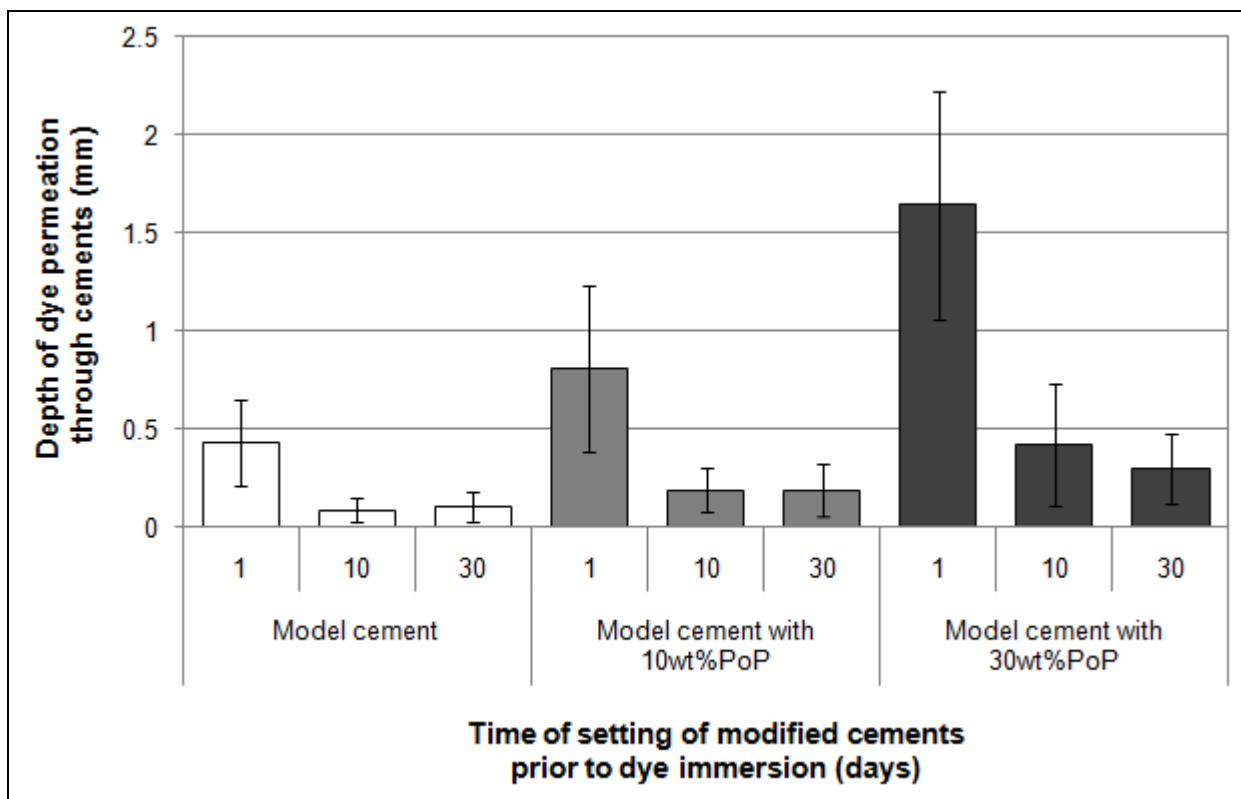


Figure 96: Crystal violet dye permeation through model cements only and model cements consisting of 10 and 30 wt% PoP. Standard deviations were represented as error bars on the graph. Cements stored for only 1 day prior to Crystal violet storage had the greatest dye permeation recorded when compared with 10 and 30 days of cement storage before dye immersion.

8.3.2 Safranin O permeation

Safranin O fully permeated cements containing PoP additions regardless of the time cements were set for before immersion (figure 97). Model cements with no PoP had the least dye permeation when set for the longest period of time (30 days) which decreased with increased setting time of model cements.

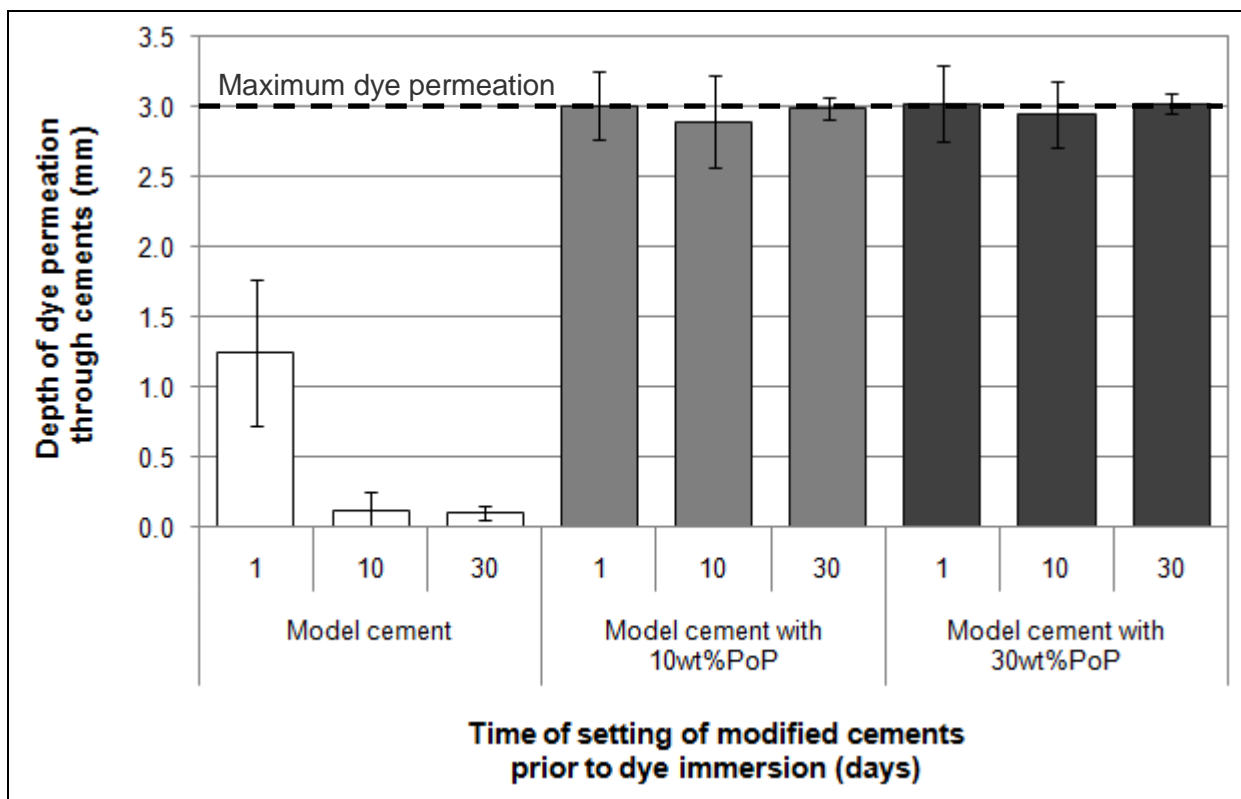


Figure 97: Bar chart to represent the depth of Safranin O dye permeation through cements with or without PoP additions and stored for varying lengths of time before immersion in Safranin O dye. Standard deviations were represented as error bars on the graph. Safranin O dye permeated through model cements the least when compared with 10 and 30 wt% PoP additions which had been permeated entirely.

8.3.3 India ink permeation

India ink permeated through cements with or without PoP additions very little (<0.1 mm) compared with other solutions of dyes/ink used for the study (see appendix 5.1 for values).

Crystal violet was selected as the dye for the study to investigate the sealing ability of model cements and MTA, and the concentration of this dye was increased to 5 % for more contrast when viewing samples that had been permeated or penetrated by dye.

8.4 *Sealing ability of model cements and commercial MTA containing PoP:*

tooth model

Dye permeation was measured as the distance the dye or ink travelled into cements from the point of contact with dye to the point dye was no longer visible and had stopped permeating into the cement. Dye penetration was measured as the movement of dye into cement from point of contact with the tooth to the point at which no more dye was visible in the cement.

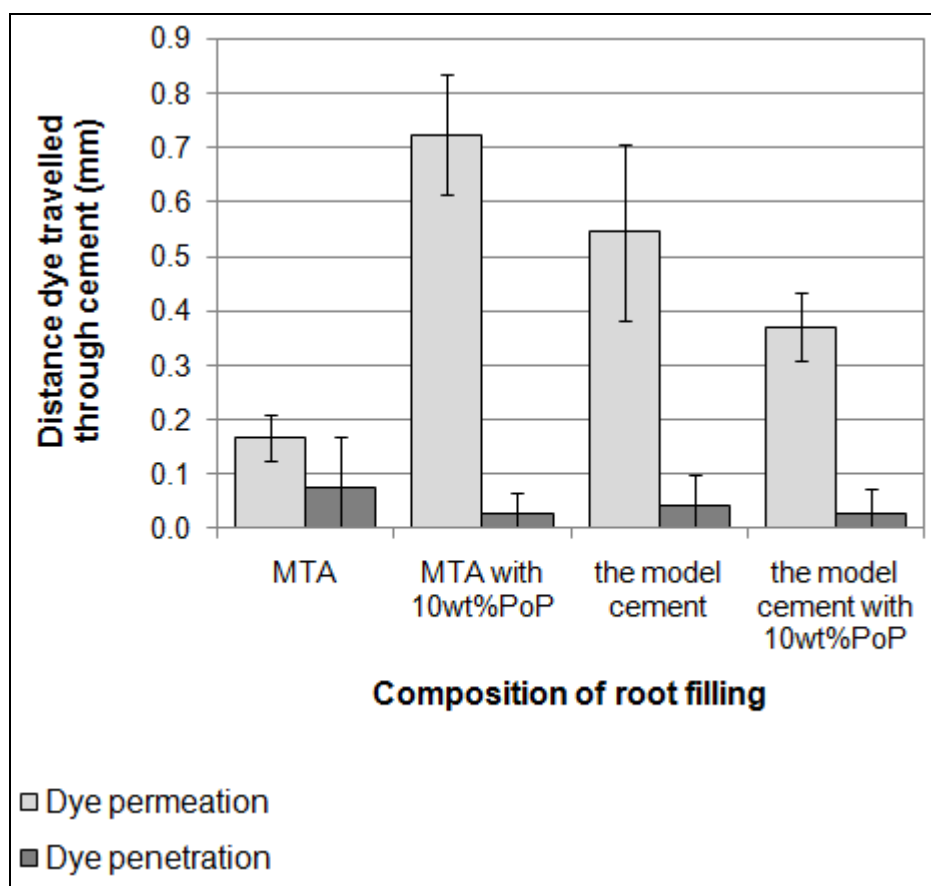


Figure 98: Bar chart to represent the dye permeation and penetration of 5 % Crystal violet into root filling cements. Standard deviations were represented as error bars for the mean values recorded on the graph. MTA containing 10 wt% PoP had the greatest amount to dye permeate through the root filling, however, did not allow dye to penetrate the cement much at all like model cement root fillings with or without PoP additions.

RESULTS

The penetration of dye into cements was significantly less ($p < 0.05$) for all cements when compared with the depth of dye permeation measured for the same cement (figure 98). Crystal violet permeated through MTA root fillings significantly less than the amount of dye permeation measured through MTA containing 10 wt% PoP and the model cement with or without 10 wt% PoP ($p < 0.05$), see figure 98. Although there was less dye permeation through MTA, dye had penetrated through MTA the most when compared with MTA containing 10 wt% PoP and the model cement with or without 10 wt% PoP (see figure 98) but no statistical differences were apparent ($p > 0.05$). The model cement without 10 wt% PoP additions had 0.54 ± 0.16 mm of dye permeation which was significantly greater ($p < 0.05$) than the amount of dye permeation measured through model cements containing 10 wt% PoP. 10 wt% additions of PoP to MTA and the model system led to the lowest values for dye penetration recorded for these root fillings when compared with MTA and the model cement without PoP additions (figure 98).

9 DISCUSSION

9.1 *Characterisation and generation of a model system for MTA*

9.1.1 **Selecting cements for use as components of the model system**

Cements with low PLRs (2.5 g/ml) tended to have increased porosity and decreased CS, with the exception of MC. The higher porosity for lower PLRs was likely to be due to the incorporation of excess water (that was not consumed in the setting reaction) being retained in the cement structure. The increased porosity could then have acted as possible flaws in the cement matrix and accounted for the observed decreased in CS. The high CS of 100 wt% MC at a PLR 2.5 g/ml may be explained by the ease of mixing the cement at this PLR that may have made the cement paste more workable thus aiding sample preparation. 100 wt% MC, SC and OPC generally followed the trend that with increased PLRs there was decreased CS and decreased RP. At high PLRs (4.0 g/ml), SC and OPC become unworkable, meaning that water could no longer deagglomerate and wet all particles to form a homogenous cement paste. Therefore dry spots formed within the agglomerates, which were more likely to be present within the cement paste of SC and OPC at high PLRs. The dry unreacted powder particles in cements that were difficult to mix may have acted as flaws within the samples, thus explaining the poor mechanical properties of MC at a PLR 4.0 g/ml and the extreme effect with SC and OPC which were unworkable for sample preparation.

Cements with a PLR 3.3 g/ml had a lower RP when compared with that of cements with a PLR 2.5 g/ml, despite the lower mechanical strength which was unusual since low RP is often associated with high CS [Fridland *et al.*, 2003]. Cements with PLR 4.0 g/ml were unworkable due to difficulties experienced with sample preparation, apart from 100 wt% MC samples. When

comparing the types of cements investigated in this thesis, 100 wt% MC was the easiest to mix, particularly when using a PLR of 2.5 g/ml, thereby making sample preparation easier which potentially could have contributed to the better mechanical properties observed when compared with other PLRs used. MC and SC were selected as components for the model systems of white and grey MTA and the use of OPC was disregarded due to undesirably lower CS during the study (see section 4.2).

9.1.2 Selecting the powder to liquid ratio (PLR) of the model system

A PLR of 3.3 g/ml was selected for use for the model system due to being similar to the PLR recommended for MTA. A PLR of 3.3 g/ml also possessed a relatively high CS accompanied by a relatively low RP required for the model cement which would be subjected to additives which were likely to alter these properties.

9.1.3 Selecting the type of bismuth oxide for use as a component of the model system

The smaller and more homogeneous particle size distribution of Bi₂O₃ Sigma might explain the slightly higher mechanical strength of the cement as the Bi₂O₃ particles may have been more evenly distributed throughout the set cement. Cements containing Bi₂O₃ Acros had larger particles which may not have been as homogeneously distributed in the microstructure of cements as Bi₂O₃ Sigma, meaning cement particles may not be able to interconnect, causing more flaws to form in the structure, contributing to the lower CS of these cements. Thus, Bi₂O₃ Sigma was selected for addition to the model system (section 3.3).

9.1.4 Changing the methodology of sample production

Samples that were left to set in the mould prior to being immersed in water for 6 h, 1 or 3 days did not show noticeable differences in CS and RP (see section 3.4). Samples immersed in water after 6 h of setting had a higher RP when compared with samples immersed after 1 day possibly due to components of the setting reaction such as unhydrated cement particles, being washed out of cements, causing the formation of more flaws in the cement structure. When samples were stored for 3 days before storage in distilled water, CS began to deteriorate slightly although the RP remained low. However, the CS and RP of cement began to deteriorate further with cement samples that had been stored in the mould to set for 7 days and then stored in distilled water. This may have been due to drying of the cement surface, causing the setting reaction near the surface of the sample to be restricted due to the absence of water.

For further sample production, throughout the study, cement samples were extracted after 6 h to 1 day of setting in the mould, as these samples possessed the highest CS and lowest RP.

9.2 *Effect of calcium sulphate additions on the model system*

9.2.1 Selecting calcium sulphates

The addition of CaSO_4 (Sigma) increased the mechanical strength of cements and had a more profound effect on accelerating the setting of cement when compared with the effect of CaSO_4 (Acros). This may be explained by the size of the particles observed in CaSO_4 (Sigma and Acros). Larger particles present in CaSO_4 (Acros) may not provide sulphate ions as readily as smaller particles for the hydration and the setting of MC which may therefore be the reason for the initial setting being five times slower than CaSO_4 (Sigma). Also, some of the CaSO_4 (Acros) powder particles resembled those of Gyp (see figure 15), CaSO_4 (Acros) therefore may have

partially hydrated to gypsum (see figure 15). The additive was also observed to have a less powerful accelerating effect compared with CaSO_4 (Sigma), since Gyp had no detectable effect on the setting of cement. From the data collected, CaSO_4 (Sigma) was selected for further study as these cements accelerated the setting of MC and improved the CS of MC when compared with the effects of CaSO_4 (Acros) on MC.

Gyp (Acros)-containing cements had the lowest CS and highest RP in comparison with Gyp (Sigma), therefore Gyp (Sigma) was used for further study. Gyp-containing cements displayed the lowest mechanical strength and highest relative porosity of all additive containing cements.

9.2.2 **5-30 wt% calcium sulphate additions to model cement (white and grey)**

Grey model cement containing 5 and 10 wt% PoP and CaSO_4 improved the CS and maintained RP whilst setting considerably faster than the MC control. This may be explained by the improved workability of the cement paste, making sample production much easier. Gyp had no detectable effect on the setting time of the cement as Gyp is less soluble than PoP and CaSO_4 [Bundesverband der Gips- und Gipsplattenindustrie, 1995]. However an accelerated setting was observed in cements containing PoP and CaSO_4 , which may have been explained by PoP and CaSO_4 being more soluble in water than Gyp (see section 5.7, figure 40).

The accelerated setting of PC, observed when the initial setting time of cement containing calcium sulphates were measured, was likely to have been caused by an abnormal or ‘false’ setting of PC (i.e. grey and white model cements), caused by the addition of the calcium sulphates PoP and CaSO_4 . The presence of excess calcium and sulphate ions may have caused a higher sulphate ion concentration in solution [Zhang *et al*, 1996], thus promoting the hydration

reactions of alite and belite [Ghosh *et al*, 2002] to form CSH and $\text{Ca}(\text{OH})_2$ phases. The hydration of alite is important for early strength development of the cement paste [Ghosh *et al*, 2002].

9.2.2.1 *Large additions of calcium sulphates to the model system*

Large (20 wt% and 30 wt%) additions of PoP and CaSO_4 to the model system accelerated the setting considerably, although generated cements with low CS and high RP (the latter increased by up to one third) as shown in section 4.3.2. This may have been due to the development of more microcracks that were observed throughout the cement structure (see figures 24 and 27) caused by the stress of rapid setting, which may compromise the material's ability to seal and also its potential longevity.

It was possible that elongated, rectangular-shaped particles (observed using SEM in 20 wt% PoP and Gyp-containing model cement) may be unreacted gypsum particles in model cement that contained large amounts of Gyp, or arose from PoP or CaSO_4 particles that had hydrated to gypsum, leading to the precipitation of gypsum crystals. The presence of gypsum crystals in the set cements (observed in SEM photomicrographs, see figures 23 and 24, section 4.3.3) was likely to weaken the structure of these cements, contributing to the lower CS observed in cements containing large additions of calcium sulphates (see figure 17). The XRD patterns supported this explanation which showed a gypsum phase present in 20 wt% PoP-containing set cements (see Figure 38).

Needle-shaped crystals were observed in model cements containing PoP and CaSO_4 (see Figure 26) that may have been ettringite crystals and were likely to have formed as a consequence of excess sulphate ions in solution [Tzouvalas *et al*, 2004]. PoP and CaSO_4 may have been more readily available in solution when compared with Gyp, due to being only

partially hydrated or anhydrous, possibly causing the rapid formation of ettringite on the outer surface of model cement.

9.2.3 **5 wt% PoP + 5 wt% CaSO₄ additions**

The addition of PoP and CaSO₄ accelerated the initial setting time of the model system and also increased the CS and RP of cements (refer to section 4.3.2) but large additions of CaSO₄ compromised the mechanical properties. However, combining both PoP and CaSO₄ in cement decreased the initial setting time more than similar single additions whilst maintaining a high CS and a low RP for the model system. Ber *et al.* (2007) combined two accelerants (MC and CaCl₂) to a PC-based material and found that the combination decreased the setting time more than just one additive, with little change in the CS. Therefore combining two additives may have been a more effective approach than adding individual components to a PC-based material for accelerating the setting time and maintaining the CS and RP.

9.3 ***Effect of PoP on the setting and mechanical properties of MTA***

The addition of PoP accelerated the setting of MTA and the grey model cement, from several hours to minutes (see section 4.3.1), without statistically significant differences in the CS and RP of MTA and the grey model system. The shortest setting time recorded from the literature, for an accelerated MTA and the model system, which contained calcium nitrite/nitrate, was 7.0 min; however, this additive combination could not accelerate the setting of white MTA as profoundly, setting in 66.7 min [Wiltbank *et al.*, 2007]. Calcium sulphates, PoP and CaSO₄, however, accelerated the setting time of PC (SC and MC) and grey MTA drastically as these cements set in less than 40 min and have a greater potential to accelerate the setting of white

MTA, when compared with calcium nitrite/nitrate [Wiltbank *et al*, 2007] due to its effect of accelerating the setting of SC (Figure 29).

The accelerated setting observed with PoP addition to MTA and the model system may have been due to ‘false’ setting of the cement paste. On hydration of MTA or the model system, an abundant, readily available supply of calcium ions are released into the cement paste, which are required for the setting reaction [Taylor *et al*, 1997] and this supply was increased with PoP addition. The excess calcium and sulphate ions, which are not required to partake in the setting reaction, precipitate out of the cement paste, causing the cement paste to stiffen. False setting is not a chemical acceleration of the Portland cement setting since it can be reversed by applying force to the stiffening cement paste through vigorous mixing. ‘False’ setting is associated with low initial mechanical strength, which is, however, not important for this material that is routinely used in non-load bearing applications. The presence of gypsum was detected by XRD in cements containing PoP, supporting the possibility that the cement paste underwent ‘false’ setting.

The model cement was affected by PoP in the same way as grey MTA as both showed accelerated setting times, in association with a decreased strength and increased porosity. The increase in porosity observed for the model system containing PoP may have been a consequence of decreased workability incorporating more air bubbles into the cement on mixing and more agglomerates forming. The decreased workability means there would have been less dispersion of the agglomerates in the cement paste, which would result in structural flaws in the cement, therefore leading to decreased CS [Coomaraswamy *et al*, 2007]. Decreased apparent density was observed for MTA and the model system with increasing PoP content which can be explained by the lower apparent density of PoP powder ($\approx 2.8\text{g/cm}^3$) when compared with that of PC powder ($\approx 3.1\text{g/cm}^3$) and slightly increased RP of cements containing PoP.

DISCUSSION

When the CSs of samples produced from smaller moulds were compared with previous studies performed using a larger-sized mould (see section 3.1.2.2), the CS for model cements without PoP was higher for samples produced using a PTFE split mould that generated samples 4x6 mm in dimension compared with samples produced using the PTFE split mould that generated samples 6 x 12 mm. A possible explanation for this would be that there are the same number of flaws present regardless of the size of the sample. These flaws may be more prominent in samples that were 6 x 12 mm (diameter, height) in dimension compared with 4x6 mm, which could have caused the lower CS observed for cements with dimensions 6 x 12 mm (diameter, height) compared with cement samples with the dimensions 4x6 mm (diameter, height) [Kendall *et al*, 1983].

The main cement chemistry, forming the major phases in the cements, was unaffected by PoP addition which has not been shown previously for other setting accelerating additives that have been introduced to MTA as XRD data has not been presented [Ber *et al*, 2007, Wiltbank *et al*, 2007, Kogan *et al*, 2006]. As the major crystalline phases in the PoP modified MTA remained unchanged and only calcium sulphate phases (e.g. gypsum) appeared as additional phases, the biological responses to the modified material are not expected to be adversely affected and should remain similar to those previously identified with respect to unmodified MTA. As the porosity of MTA was unaffected by the addition of PoP can also be expected that there would be no deterioration in sealing ability of the material.

SEM revealed that cements containing PoP contained large hexagonal plates (see figure 37) which were also observed by Tingery *et al*. (2008) when grey and white MTA was set in the presence of distilled water. These crystals may have been monosulphate crystals [Griesser *et al*, 2002] caused by ettringite consumption, possibly showing that the quantities of hydration products of the cement could have been altered as well as the initial setting time of these cements

DISCUSSION

being reduced (see Figure 2 showing the phases formed with time). Alternatively, the large hexagonal particles could have been calcium hydroxide crystals that had formed at the outer surface of cements in contact with water, since calcium hydroxide is a product produced during the hydration of PC [Taylor *et al.*, 1997]. There was a considerable difference between the outer and fracture surfaces of MC-model cement and MTA, possibly due to the fact that at the outer surface, the cement was exposed to more water than at the fracture surface, meaning that the setting of MTA depends on the environment in which it set. The setting environment could be important in determining the surface properties of MTA when placed within a root because the conditions under which it sets are likely to affect the properties of the material. The reaction of bone cells or tissues to MTA set *in vivo* may affect the behaviour of these cells and subsequently the attachment of these cells to the cement surface.

9.4 *Sterilisation of powders and set cements*

Dry heat treatment of model cement powders, wet or dry autoclaving of the set model cements caused the CS of model cements and model cements containing 10 wt% PoP to decrease significantly ($p < 0.05$) and the RP increased considerably (see results section 5). However, there were no significant differences ($p < 0.05$) in the CSs of 30 wt% PoP-containing model cements that had or had not been sterilised but RPs increased when cements containing 30 wt% PoP were sterilised. Dry heat treatment may have caused decreased CS due to the increased RP, weakening the cement structure. The increased RP observed for cements that were autoclaved wet or dry may be a result of the removal of water bound in the cement matrix. The water removed from the cement matrices may have caused increased porosity and an increased number of unhydrated particles and since the hydration reactions were not fully completed this may have caused a weakened cement structure as indicated by the decreased CS.

Other researchers have used various methods in order to ensure sterility of MTA and PC for placement in cell cultures. Ethylene oxide gas and UV irradiation have commonly been used with the aim of sterilising MTA and PC [De Deus *et al*, 2005], however, other methods of sterilisation have included the use of ethanol, which in actual fact would not sterilise samples completely should they contain bacteria.

9.5 *Long term stability and solubility of PC containing PoP*

9.5.1 **Water storage**

9.5.1.1 *Grey model cements*

The CS of MC model cements with or without PoP additions, stored for 10 and 30 days were similar and the RP of model cements decreased with increased setting time for model cements and cements containing 10 wt% PoP only up to 3 months of water storage. After 3 months of storage the CS of model cements with or without 10 wt% PoP increased significantly when compared with 10 and 30 days, but remained the same for cements containing 30 wt% PoP. A possible explanation for the weaker cement structure at 10 and 30 days was that the hydration of alite occurs first when cement powders hydrate. Alite from PC hydrates in the presence of water and forms initially thus strengthening the cement paste before the structure becomes more rigid from the hydration of the belite phase that usually occurs one month after the initial hydration of the cement [Taylor *et al.*, 1997]. The high concentration of calcium ions released from cements at week 4 confirmed that the hydration of alite was complete after 5 weeks since alite hydration yields three times more calcium hydroxide as a product compared with that of belite [Lea *et al.*, 1970, Bye *et al.*, 1999] and the concentration of calcium ions released into solutions from cements was highest at 4 weeks and dropped sharply by 5 weeks [see figure 51]. RPs for model cements with or without 10 wt% PoP decreased slightly for samples stored for 10 days to 3 months. The strength increase observed for cements stored for 3 months and also the increased release of calcium hydroxide (observed by the measurement of high Ca^{2+} ion concentration, see section 6.9.1) may be due to the hydration of belite, forming CSH, a rigid

structure, responsible for long-term strength of the cement [Taylor *et al*, 1997] as described in section 1.4.2.2.

The pH of the water the cements were immersed in increased to above pH11.2 for unmodified and PoP-modified grey model cements and remained that high during the 12 month study. This was due to the calcium hydroxide produced by the hydration reactions of PC, dissociating in aqueous solution to calcium and hydroxyl ions, which then resulted in the increased pH and high concentrations of calcium ions measured in the storage solutions [Santos *et al*, 2005]. The results also corresponded with the findings by Fridland *et al* where it was reported that the grey MTA had increased the pH of the water it was immersed in for 72 days to pH11.6 [Fridland *et al*, 2005]. Increasing the local tissue pH, however, has the potential to cause zones of tissue necrosis by altering the integrity of the cytoplasmic membranes of individual cells and changing the chemical composition and thus causing the denaturation of organic components required to support tissue growth [Estrela *et al*, 1999]. However, the release of calcium ions causing the increased pH may have a positive effect on the repair of tissues, for example by the activation of alkaline phosphatase [Estrela *et al*, 1999]. The response of cells to MTA and the model cement will be later discussed in section 9.6. Antibacterial and microbial action may prevail in modified and unmodified cements stored for up to 12 months due to the high pH caused by the production of calcium hydroxide which may cause the prevention of bacterial growth surrounding the root filling cement, an important requirements for the success of root filling cements [Chong *et al*, 2005].

Model cements and cements containing 10 wt% PoP were mechanically stable at 6 and 12 months despite a slight increase in RP. The mechanical stability was likely to be due to the continued belite reaction occurring in the cement matrix, which was responsible for the long-term mechanical strength of the cements. However, large additions of PoP (30 wt%) appeared to

DISCUSSION

drastically decrease strength of the material, as indicated by the reduction in CS and increase in RP with storage time and also by the pores that were apparent in the SEM photomicrographs of the cement structure. This can be explained firstly by an interference of large quantities of PoP with the setting reaction of PC, as indicated by the measured decreased specific density (see results chapter 6.8), causing the cement to ‘false’ set upon hydration due to the formation of a weak gypsum sub-structure within the cement matrix. The gypsum substructure, formed through the precipitation of PoP would be likely to be more soluble and therefore dissolve out of the cement. The PoP would also not be consumed during the hydration reactions as calcium sulphate would be in excess due to the large addition of PoP to the cement. The consequence of gypsum dissolving out of the cement structure is thus likely to have caused the low mechanical strength and increased porosity observed in model cements containing 30 wt% PoP with increased length of water storage. The measured reduction in dry density with storage time also explains the decreased CS and increased RP recorded for 30 wt% PoP-containing cements (see results chapter 6.8).

Fracture and outer surfaces of the grey model cements differed considerably as small, needle-like particles were present on the outer surfaces of the grey model cements but were not apparent on the fracture surfaces. These small needle-like particles may be C-S-H crystals having formed as a result the continuing hydration of the cement surfaces in water which caused the cement surface to be hydrated further and thus causing the release of the newly formed calcium hydroxide from the cement surface [Lea *et al*, 1970].

9.5.1.2 *White model cements*

The long term mechanical stability of white and grey model cements with or without 10 wt% PoP additions were not affected by water storage since CS and RP did not change noticeably for unmodified and 10 wt% PoP-modified white cement that were stored in water for up to 9 months. The CS was expected to follow the trend shown for grey model cements, however, no significant decrease in CS with storage time was found. However, the mechanical stability was affected by 30 wt% PoP additions to white cements after 6 months when CS decreased and RP increased. Pores were evident in the outer surfaces of the cements with or without PoP whereas none were present in the fracture surfaces observed using SEM (see results chapter 6.8). Therefore, decreased mechanical stability cannot be explained by the dissolution of the gypsum out of the cement structure. Since no porosity was evident in the fracture surface of the cement, the explanation for reduced mechanical stability may have been due to the weak structure of gypsum that led to lower CS in these cements containing 30 wt% PoP additions.

9.5.2 **Culture medium storage**

The mechanical stability of grey model cements were unaffected by long term storage in culture medium. Grey model cements with or without 10 wt% PoP had a higher mechanical strength compared with 30 wt% PoP-modified cements when stored for 9 months. The structure of 30 wt% PoP-modified cement had a less compacted structure when observed by SEM and measured using helium pycnometry than grey model cements with or without 10 wt% PoP in culture medium. A possible explanation for this is that PoP has a lower specific density ($\approx 2.8\text{g/cm}^3$) compared with PC ($\approx 3.1\text{ g/cm}^3$) therefore 30 wt% PoP additions to grey cements would contain less PC and more PoP than the model cement, thereby causing reduced density.

DISCUSSION

Gypsum formation in the structure of cements containing 30 wt% PoP had been observed previously in shorter studies (section 4.2.2) which has a lower CS compared with PC, therefore, large quantities of PoP in the cement would be likely to weaken the cement structure, which was demonstrated at 9 months when unmodified model cement was compared with 10 wt% PoP-modified cements.

Grey and white cements with or without PoP additions stored in culture medium for 9 months had small cement particles present on the outer surface which were not apparent from SEM micrographs of the fracture surfaces of these cements or the outer surfaces of either PoP-modified and unmodified grey or white cement that were stored in culture medium or distilled water for 10 days. Tingery *et al.* (2008) analysed the structure of white and grey MTA, set in the presence of foetal bovine serum and distilled water. It was reported that a globular structure formed on the surfaces of white and grey MTA in contact with foetal bovine serum that was not present on MTA stored in distilled water, which instead had large hexagonal crystals present. Sarker *et al.* (2005) reported that calcium ions were released from MTA when stored in phosphate buffered saline that led to the precipitation of hydroxyapatite crystals on the surface of MTA and white PC [Coleman *et al.*, 2007]. In another study, it was found that a thick apatite coating formed on the surfaces of unmodified and modified tetrasilicate based white PC when stored in Dulbecco's Phosphate Buffered Saline for 2 months [Taddei *et al.*, 2009b] and after storage in a Hank's balanced salt solution [Taddei *et al.*, 2009a]. The crystal precipitates on the surfaces of grey and white cements stored in culture medium stored for long periods of time resembled the precipitates on the surface of MTA reported in the literature [Sarkar *et al.*, 2005, Reyes-Carmona *et al.*, 2009] and may indicate the precipitation of hydroxyapatite. The formation of an apatite layer, covering pores and defects in the root filling material, may be responsible for the chemical bond that forms between MTA and the dentinal walls that may have promoted the

use of MTA over other root filling materials [Reyes-Carmona *et al*, 2009]. Therefore, PoP-modified grey and white cements may have the potential to encourage faster hydroxyapatite deposition on the surface, which could be a biologically active substrate for the colonisation of cells and improve the longevity of the root filling.

9.6 *Effect of modified model cements and MTA on different cell cultures*

Primary osteoblastic and periosteal cultures were used since these cells originate from bone which may be considered similar to odontoblasts that are primarily present in dentine and fibroblasts which could be found in the dental pulp of the tooth and osteoblasts are present in the alveolar bone. Neonatal and adult BMSCs are mesenchymal cells and were used because of the osteogenic nature of these cells and since mesenchymal tissue is present in the dental pulp, therefore root filling materials are likely to come into contact with these cells.

9.6.1 **Cements in periosteal and osteoblast cultures**

Cell counts taken for periosteal and osteoblastic cultures yielded a typical growth curve. The lag phase (the establishment of the cell culture, where cells are adapting to the new environment placed within) for periosteal cultures with or without model cements was apparent from 0-4days of culturing whilst only 0-2 days for osteoblastic cultures that did or did not contain model cements. However, periosteal and osteoblastic cultures proliferated at a slower rate when containing modified or unmodified model cements compared with periosteal and osteoblastic cultures only (refer to results sections 7.1 and 7.2). Jaunberzins *et al*. (2000) reported that the effect of released calcium hydroxide in early subcultures may have had an inhibitory effect on primary osteoblasts ability to synthesise collagenous and non-collagenous proteins, thus,

DISCUSSION

potentially resulting in a slower proliferation rate, since proteins are required for the production and recruitment of new cells. This may explain why cultures containing cements had lower cell densities compared with control cultures since it was reported that PC released calcium hydroxide during hydration of the cement [Lea *et al.*, 1970].

PoP additions to model cements in cultures containing periosteal or osteoblastic caused the rate of cell proliferation to increase slightly with time when compared with unmodified model cements, though these differences were not significant. This correlates with reports that in later subcultures of primary osteoblasts exposed to calcium hydroxide increased protein production which could have gave rise to increased cell proliferation [Jaunberzins *et al*, 2000].

Cell lysis was observed directly surrounding cements within osteoblastic and periosteal cultures but was not demonstrated in the form of SEM micrographs when using osteoblastic and periosteal cultures during this thesis, which may explain the decreased cell densities observed for model cement containing cultures, with or without PoP addition. This observation was similar to what was reported in another *in vitro* study involving fibroblasts that were in contact with MTA, where lysis and medium protein denaturation was observed which was explained by the high pH of MTA during setting [Haglund *et al*, 2003].

Cell densities were significantly lower ($p < 0.05$) for cultures containing model cements and 30 wt% PoP-model cement for both periosteal and osteoblastic cultures when compared with periosteal and osteoblastic cultures without cements throughout the study. Also only very few osteoblasts were observed to be attached to model cements and 30 wt% PoP-containing cements and the number of osteoblasts attached was not consistent with time. Although no numerical values were provided for cell attachment to MTA in a study conducted by Pérez *et al.* (2003) it was observed that osteoblasts and MG-63 osteosarcoma cells attached to ProRoot MTA and mineralisation was evident. The inconsistency in the number of osteoblasts attached to model

cements with or without PoP may be attributed to the high alkalinity of MTA. It may also be possible that the pH of the material had slightly inactivated the trypsin used to remove attached cells from the surface of the cements since the number of cells counted was always very low and the trypsin changed colour to a purple shade when in contact with cements.

9.6.2 Effect of model cements on neonatal and adult BMSC cultures

9.6.2.1 *BMSCs seeded onto model cements into different volumes of medium*

Neonatal or adult BMSCs were seeded into small and larger volumes of culture medium and seeded directly onto cements and culture plates in order to see the effect of the alkaline cements (the model cement and cements containing 30 wt% PoP) on cell survival and proliferation. When the root filling material is applied directly to the root, the material will cause the surrounding tissue pH to alter, thereby having an effect on the survival of cells in direct contact with the material. Adult and neonatal BMSCs seeded onto cements with or without PoP additions showed significantly decreased cell densities with increased time when compared with cultures containing adult or neonatal BMSCs only when seeded in a small volume of medium. Cells seeded onto cements in such a small volume of culture medium could indicate what might be happening to cells comprising the tissues surrounding MTA when immediately placed in the root canal. The anti-proliferative effect of model cements with or without PoP may have been due to the high alkalinity of the cement substrates affecting the ability of the cells to survive in cultures containing the cements [Torabinejad *et al*, 1995c]. Model cements with or without PoP released large amounts of calcium and hydroxide (OH^-) ions that increased the pH of the culture medium which the cells were seeded into, causing death and damage to many cells. Therefore when the rest of the culture medium was seeded into culture wells containing model cements with

DISCUSSION

or without PoP, there may have been a large percentage of non-viable neonatal or adult BMSCs in the final volume of medium and with time these non-viable cells would release lactate dehydrogenase, thus preventing further cell proliferation [Butler *et al*, 2004].

In contrast, adult and neonatal BMSCs that were seeded onto cements in a larger volume of medium had higher cell densities when compared with cells seeded onto cements in smaller volumes of medium. However, model cements with or without PoP showed a slight anti-proliferative effect on cells as the cells that proliferated in culture wells containing cements at a slower rate when compared with the control (adult or neonatal BMSCs only). Cell proliferation increased with increased time in culture for cultures that had cells seeded onto model cements with or without 30 wt% PoP in a large volume of medium, which differed to cultures that had cells seeded onto model cements (with or without 30 wt% PoP) in a small volume of medium. The reason for the increased cell proliferation with time, which was unlike the effect observed when cells were seeded onto cements in smaller volumes, may be due to the calcium and hydroxide ions being released from cements into a larger volume of medium therefore the concentration of the alkaline hydroxide may have been higher for cells seeded onto cements in a small volume of culture medium. The buffer, HEPES, within the culture medium used may also be responsible for balancing the pH of the medium once calcium and hydroxide ions were released from cements. Cultures containing a larger volume of medium at the time cells were seeded onto cements (and as a consequence more HEPES buffer) would be likely to have little effect from the released calcium and hydroxide ions since the effects would be diluted. Therefore, little effect on the attachment and proliferation of adult and neonatal BMSCs, unlike culture that contained less culture medium at the time cells were seeded onto cements.

Cultures containing cements with 30 wt% PoP had higher cell viabilities than cultures containing model cements only, which increased with culture time. PoP has been used in

DISCUSSION

medicine *in vivo* as it was found to encourage bone regeneration through the release of Ca^{2+} ions [Orsini *et al*, 2004]. Therefore it may be possible that the addition of PoP to model cements could promote neonatal or adult BMSCs to proliferate at a slightly higher rate than in unmodified model cements.

No cells were found to have attached to model cements after 2 days when neonatal or adult BMSCs were seeded onto cements in a small volume of medium but neonatal and adult BMSCs attached initially to model cements that contained 30 wt% PoP. Model cements with 30 wt% PoP additions may have been a more suitable substrate for cell attachment after setting for 4days (and only 3 days in cultures as cements were set for 24 h prior to placement in cultures) than model cements only as the microstructure was less compact structure but had fewer imperfections on the surface of the cement potentially due the accelerated hardening caused by PoP additions. PoP is responsible for providing an excellent biological response when in contact with bone cells [Orsini *et al*, 2004] and therefore may be responsible for increased initial cell attachment observed in cultures containing model cements with PoP additions when compared with cultures that contained model cements without PoP.

Fewer neonatal and adult BMSCs attached to model cements (with or without PoP) when seeded onto cements into a small volume of medium when compared with a larger volume of medium. The alkaline pH of model cements with or without PoP could have been decreased to a pH where cell survival and attachment was more likely should the cement be surrounded by a larger volume of culture medium compared with smaller volumes, therefore potentially encouraging the attachment of cells to the surface of the cements.

9.6.2.2 *Cements placed onto established BMSC cultures*

Adult BMSCs demonstrated inhibition zones of cell growth and cell death when in contact with MTA, MTA with 10 wt% PoP, model cements with and without 10 or 30 wt% PoP. Similarly, in previously published studies, zones of cell lysis and death were apparent when L929 mouse fibroblasts were observed surrounding grey and white MTA [Miranda *et al*, 2009]. White and grey MTA, set for 48hours, were placed into L929 fibroblast cultures and cultured for 48hours. This study found MTA to be slightly cytotoxic, with grey MTA being less cytotoxic than white MTA to L929 cells and there were also zones of cell lysis, dead or loosely attached cells surrounding MTA and only furthest away from MTA were viable cells found [Miranda *et al*, 2009]. In another study it was reported that MTA caused a layer of necrotic tissue in dog pulps [Faraco *et al*, 2001]. The studies conducted by Faraco *et al*, 2001 and Miranda *et al*, 2009 support the finding of the present study which demonstrated that cell lysis was also occurring surrounding model cements and MTA due to the initial cytotoxicity of these materials, which may have been caused by the release of calcium and hydroxide ions and that this might have happened also explaining the zones of cell growth inhibition.

However, BMSC cultures containing model cements with or without PoP additions showed increased cell density with time, though the cell proliferation rates were lower than for the control. Other studies reported that the initial cytotoxic effects that were evident when endothelial cells were in contact with the high surface pH of MTA which resulted in the denaturation of medium proteins and cells adjacent to cement [de Deus *et al*, 2005] and beneath the cement [Saidon *et al*, 2003]. This toxic effect was reported to be a concern as damage and irritation could cause degeneration of periapical tissues, thus delaying wound healing [de Deus *et al*, 2005]. The cytotoxicity of MTA decreased gradually over the experimental time period

allowing the cell culture to recover [Saidon *et al*, 2003, de Deus *et al*, 2005]. These results support those previously reported in the literature [Estrela *et al*, 2000, Holland *et al*, 2001a, Abdullah *et al*, 2002, Saidon *et al*, 2003]. The high alkaline pH of MTA decreased with time, causing the deterioration of L929 mouse fibroblasts to be reduced by the high pH [Saidon *et al*, 2003]. Tronstad *et al*, 1981 reported that an increase in pH combined with the availability of Ca^{2+} and OH^- ions affected the enzymatic pathways of cells and the mineralisation process of dental hard tissues exposed to calcium hydroxide. Therefore, it may be assumed that the slow proliferation rate of BMSCs in cultures containing modified model cements and MTA may have been a consequence of a high concentration of calcium hydroxide released into culture medium that could cause damage to cell membranes that would need to be repaired [Tronstad *et al*, 1981]. Calcium hydroxide may have dissociated to result in high concentrations of Ca^{2+} and OH^- ions [Tronstad *et al*, 1981], the latter could have had an inhibitory effect on protein synthesis [Jaunberzins *et al*, 2000] delaying repair of damaged cells. With time, the cultures would have had the chance to recover due to the dilution of calcium and hydroxide ions, caused by changing the medium of cultures that would decrease the high alkalinity of culture medium in MTA cultures [de Deus *et al*, 2005] and the decreased release of calcium and hydroxide with time (>30 days), observed in figure 51, caused by the hydration of belite in PC ions (calcium hydroxide being the product of hydration) [Lea *et al*, 1970].

9.6.2.3 *Cell attachment to modified model cements and cement pastes*

There were more adult BMSCs attached to culture wells compared with the density of cells attached to model cement samples and pastes with or without PoP additions. Model cements with or without PoP may be slightly cytotoxic as of the cells attached to cements, only grey model cements showed viable adult BMSCs attaching to its surface and only viable, non-adherent

cells were found in culture medium (see section 7.4.4 and 7.4.5). However, grey unmodified model cement, white model cements either modified or unmodified showed non-viable cells attached and mostly non-viable, non-adherent cells were found in the culture medium. The pH of rat bone marrow cell cultures increased to 7.5 when MTA was added to cultures with adult BMSCs in a similar study [Nakayama *et al*, 2005]. However, the pH of the culture medium from the cultures containing grey and white unmodified model cements was much higher than the pH of adult BMSC cultures only which could explain the loss of viability in cultures containing grey or white model cements, since cells require a pH between 7.2 and 7.5 to sustain adherence to a substrate and to maintain function of the cells [Butler *et al*, 2004]. The presence of non-viable cells in culture medium from cement-containing cultures may also be attributed to a high concentration of calcium and hydroxide ions in culture medium, which could have resulted in the destruction and damage of cell membrane proteins.

9.6.2.4 *Effect of placing pre-washed cements in adult BMSC cultures*

Cultures containing cements that had been washed or wetted before placement into cultures showed reduced density of viable cells attached to the culture wells containing these cements (apart from ethanol washing of cements), but the overall viability of cell cultures containing model cements varied slightly with the solution which it was washed in, but these results were not statistically significant. Storage of cement samples in solutions before placement into culture wells does not mimic the *in vitro* situation, however, researchers have used such storage solutions to sterilise samples or simply to prevent the material from drying out.

MTA which was stored for 3 days in culture medium caused human dental pulp cells to have increased proliferation when cultured using an indirect method [Takita *et al*, 2006]. However, fibroblastic cell lines experienced an anti-proliferative effect from indirect contact with

unwashed MTA, set for 2 days before culturing [Koulaouzidou *et al*, 2005] which was confirmed by the findings of the present study. The proliferative effect observed in the study observed by [Takita *et al*, 2006] was not shown when model cements were pre-washed with culture medium as used during the present study. The storage of MTA in culture medium could have been the cause for the proliferative effect observed in the study conducted by Takita *et al*. (2006) rather than a proliferative effect being caused by MTA. Torabinejad *et al*. (1995c) reported zones of lysis surrounding fresh and unwashed set MTA when L929 fibroblast mouse-derived cells were cultured. Unwashed PC and MTA had similar cytotoxicities when exposed to human-derived endothelial cells [De Deus *et al*, 2005]. However, no cytotoxicity was observed for 1 and 7 day old human osteogenic sarcoma (U₂OS) cultures exposed to MTA, washed in McCoy's culture medium for up to 1 week and high survival rates (>100%) were reported [Kao *et al*, 2006]. Therefore, the pre-washing of cements before culturing has been considered questionable since the authors have reported different findings dependent upon the treatment of cement samples prior to placement in culture.

9.6.3 Comparing the cell types used for the study

Periosteal cultures proliferated at a higher rate compared with osteoblastic cultures which may have been due to periosteal cultures containing a higher proportion of fibroblastic cells. However, neonatal BMSCs proliferated at a slower rate compared with adult BMSCs. It would have been more likely for neonatal BMSC cultures to have the highest cell densities rather than adult BMSCs. A possible explanation for the higher cell densities of adult BMSCs cultures may be that the tissue containing these cells were harboured from tissues were removed immediately after the rat had been sacrificed but neonatal tissues containing BMSCs were harvested an hour

after the animals were sacrificed, meaning that there was a greater chance that these cells may have decreased cell viability. Osteoblastic, periosteal or neonatal BMSC cultures containing model cements that were modified or unmodified with PoP had only half the number of cells compared with control cultures. Adult BMSCs appeared to be affected the most by the addition of modified or unmodified model cement since cell number decreased to almost one quarter the number of adult BMSCs in cultures without cement, compared with osteoblastic, periosteal and neonatal BMSC cultures, see results sections 7.1-7.4.

9.6.4 Effect of modified MTA on adult BMSC cultures

Adult BMSCs were chosen for studies involving the use of commercial MTA because of the amount of preliminary studies undertaken during this thesis with adult and neonatal BMSCs. Primary cells should be used since these cells may better simulate the *in vitro* situation and thereby provide a more reliable insight into the effect of MTA and model cements containing PoP *in vitro*. MTA and model cements had similar effects on adult BMSCs and PoP additions had little effect on cell density when compared with unmodified MTA and model cements. There were fewer cells attached to cultures wells containing MTA and model cement with and without 10 or 30 wt% PoP throughout the study when compared with the control.

When cultures contained MTA and model cements, it was observed that the pH of the culture medium in close proximity to the cement increased as evident by the colour change indicated by the phenol red pH indicator present in the culture medium. The increased pH was likely to have been caused by the release of calcium and hydroxide ions from PC into the medium of cultures containing model cements and MTA with or without PoP additions. Model cements, with the principle component being PC, released calcium hydroxide when hydrated [Lea *et al*,

1970]. The particular reaction that occurs to produce calcium hydroxide is the hydration of alite which is responsible for producing a rigid structure that causes the initial setting of the cement paste and this reaction continues up until 30 days after initial hydration of the cement [Taylor *et al*, 1997].

Nakayama *et al*. (2005) reported that the pH of rat bone marrow cell cultures increased with cultures containing MTA, but reported that the effect of the pH increase lead to low cytotoxicity of MTA compared with other root filling materials that were investigated. Other proposed additives [Bortoluzzi *et al*, 2006] such as CaCl_2 that were been studied have shown to reduce the pH of MTA when added, therefore compromising the properties of MTA such as its antimicrobial potential and may also cause a change in the local tissue pH if applied *in vivo*.

Cell proliferation increased for cultures containing modified and unmodified MTA and model cements with increased time in culture, but cultures did only have half of the cell density of control cultures. MTA and model cements with or without PoP may affect cells in the same way calcium hydroxide *in vitro* using a similar mechanism of action on cells [Holland *et al*, 2001a]. Estrela *et al*. (2003) reported that MTA encouraged the deposition of hard tissue using a similar mechanism to calcium hydroxide. Estrela *et al*. (2003) explained that the mechanism of action of calcium hydroxide is associated to its capacity to activate tissue enzymes, to encourage tissue regeneration and repair through mineralization. Estrela *et al*. (2003) described that the activation of alkaline phosphatase (a hydrolytic enzyme) induces mineralisation [Granstrom *et al*, 1972] by the dissociation of hydroxide ions from calcium hydroxide, which results in elevated pH [Tronstad *et al*, 1981]. Alkaline phosphatase works best between the limits of pH8.6 to 10.3 [Thompson *et al*, 1966] and has the capacity to separate the phosphoric esters, freeing phosphate ions which react with calcium ions to form a precipitate, calcium phosphate, the molecular unit of hydroxyapatite [Estrela *et al*, 2003].

DISCUSSION

Adult BMSC cultures containing model cements or MTA had a greater impacted on cell proliferation throughout the studies conducted during this thesis. Nakayama *et al.* (2005) reported that rat bone marrow cell cultures containing MTA proliferated at a slower rate compared with control cultures without MTA and that the reason for the decreased cell proliferation rate could be due to the high concentration of Ca^{2+} and OH^- ions released from MTA into culture medium which was investigated by Orii *et al.* (1999) with osteoblast-like cells. High concentrations of Ca^{2+} ions, which correlated directly with the concentration of OH^- ions, were reported when model cements and cements containing PoP were stored in water (see section 4). Therefore it may be likely that the release of calcium hydroxide, a product of the hydration reaction, is causing the slower proliferation rates of cultures containing cements compared with BMSC cultures without cements due to high concentrations of OH^- ions released into culture medium.

The benefits of PoP additions to model cement and MTA have not yet been fully revealed within this thesis. PoP additions to model cements and MTA in cultures containing adult BMSCs caused the rate of cell proliferation to increase slightly with time compared with unmodified MTA or model cements, though these differences were not significant. Calcium hydroxide is obtained through the hydration of calcium oxide [Greenwood *et al.*, 1984]. It had been reported that calcium hydroxide, used as a pulp capping material, promoted the formation of hard tissue bridges for the protection of the dental pulp and had an antimicrobial action to prevent the growth of bacteria that may be present when used for endodontic treatment as a sealer or capping material [Estrela *et al.*, 2003]. Like calcium hydroxide, the high pH of both model cement and MTA (unmodified and PoP-modified) may have the capability of activating tissue enzymes that favour tissue repair through mineralisation [Estrela *et al.*, 2003]. Cell viabilities were highest for modified and unmodified model cements compared with MTA with and without 10 wt% PoP at 14days.

DISCUSSION

Cell lysis, dead or loosely attached cells were observed surrounding MTA and only furthest away from MTA were viable fibroblast mouse-derived cells found [Saidon *et al*, 2003, Miranda *et al*, 2009]. These reports were confirmed when a zone of cell growth inhibition was observed on culture wells surrounding model cements and MTA (unmodified or PoP-modified) but the least amount of cell lysis was observed around unmodified MTA. However, the size of the zone of cell growth inhibition cannot be compared with the findings of the present study since the authors did not measure the distance to the cement at which cells began to grow normally [Saidon *et al*, 2003]. MTA particles were reported to be smaller in size and more uniform in shape compared with the particle size of PC [Damaschke *et al*, 2005], which means that the density of the material would differ if the same sized samples were compared. The density of MTA was reported to be higher than the model cement during this thesis. The increased amount of cement that comprised an MTA sample compared with PC-model cements may generate more calcium hydroxide whilst setting, which is likely to cause a change in the cultures pH, which could damage cells and cause cell death. Despite releasing more hydroxide ions than PC-model containing cultures, MTA containing cultures showed the least cell lysis since the zone of cell growth inhibition was also smaller compared with other cements.

No adult BMSCs attached to model cements or MTA with or without 10 wt% PoP additions and hence were not reported in the results section (see section 7.4.7). Camilleri *et al*. (2005b) found that the soluble components of MTA (achieved via indirect contact of the cells with MTA) had a proliferative effect on human osteosarcoma cell lines after 24 h but direct contact of these cells with MTA resulted in decreased cell viabilities. However, in 24 and 72 h studies that used human gingival fibroblasts, there were significantly lower cell viabilities in cultures that had indirect contact with MTA compared with the control [Gwen *et al*, 2007]. It

may therefore be speculated that MTA had a negative effect on adult BMSC adherence during this study due to the high pH of the material.

9.7 *Sealing capabilities of modified MTA and the MTA model cement*

9.7.1 **Development of a tooth model: dye permeation studies**

9.7.1.1 *Method development*

Dye permeation was measured using a grid to prevent measurements from being subjectively made and became a more reliable method to use when compared with measuring dye permeation without a grid since the method was standardised therefore measurements were made in fixed positions and measured more accurately. The dyes studied permeated to different depths in the cement samples and also permeated differently at various sites in the cement sample. Rhodamine B permeated through cements more than all other dyes or inks (for instance with cements set for 3 and 10 days before dye immersion) and almost permeated cements completely (cements set for 1 day before dye immersion) which was more apparent when high concentrations of dye were used. According to Francci *et al.* (1995) the use of Rhodamine B simulates the leakage of toxins and enzymes produced as a result of bacterial metabolism [Bortoluzzi *et al.*, 2006] and therefore could be used for the comparison of dye leakage into model cements and MTA. However, as illustrated by the dye permeation studies, Rhodamine B almost permeated the entire sample therefore comparative analysis of dye leakage through unmodified and modified model cements and MTA was impossible.

Safranin O had not been used by other researchers for the evaluation of the sealing ability of MTA using the dye leakage method but had been used for the investigation into the

DISCUSSION

penetration of propylene glycol into dentine [Cruz *et al*, 2002]. However, it was chosen for the present study due to having a lower molecular weight ($M_r=350.9$) when compared with the molecular weight of Methylene blue ($M_r=373.9$) for example. Dyes with lower M_r are likely to have increased dye permeation into cement. Safranin O almost permeated the entire cement samples and therefore would be inadequate for use in a tooth model since the control would be fully saturated with dye and thus no comparison could be made with cements containing additives. Safranin O is a very bright contrasting colour against the colour of the cement which is a shade of grey. However, when Safranin O permeated into cement; it formed what almost appeared to be a double line of dye permeation, like a two tier permeation of the dye, which makes measuring the actual permeation of the dye confusing to evaluate. The permeation of Safranin O did not permeate into cements homogeneously as some areas showed more dye permeation than others, unlike the permeation of other dyes used.

Dye permeation could be measured more easily when 2 % Crystal violet was used, compared with other dyes used as the boundaries between where the dye had permeated into cements and the area that did not show dye permeation was clearly visible and thus Crystal violet was selected for use in the study due to the contrast of the colour of the dye to the colour of the cement and due to its use in existing dye leakage studies [Goldman *et al*, 1989].

Toluidine blue was selected on the basis that this dye had almost the same molecular weight ($M_r=373.97$) as Methylene blue ($M_r=373.9$) and appeared to be similar in colour. Methylene blue and Toluidine blue did not contrast well with model cements causing difficulty in determining the depth of dye permeation. This may be due to the interaction of Methylene blue dye with calcium hydroxide released from the cement during the hydration reactions of calcium oxides, which is known to cause decolouration of the Methylene blue [Wu *et al*, 1998] that may

have attributed to inaccurate and misleading results as found during a dye leakage study [Souza *et al*, 2009].

India ink did not show any permeation into cements but had been proposed as its particles are similar in size to bacteria [Schäfer *et al*, 2002, Mente *et al*, 2009] hence the reason for its investigation as a potential ink for the determination of the sealing ability of cements with PoP additions.

9.7.1.2 *User dependence of the method for determining dye permeation*

Crystal violet and India ink permeated into cements similarly according to two users that measured the dye permeation using a grid. The dyes, Crystal violet and India ink, demonstrated the least dye permeation into cements and were the most clearly visible dyes compared with the other dyes that were investigated (see figure 91). Therefore both were chosen for further permeation investigations dye into cements with varying PLR and various amounts of additives to the cement. The other dyes that were used for the measurement of dye permeation revealed variations in measurements between the two users and therefore were not used for further investigations.

9.7.2 **Effect of PLR on dye permeation into cements**

Dye permeation increased with decreasing PLR and decreased with increased setting time of the cements before immersion in Crystal violet or Safranin O (see figures 92-95). Dye permeation increased with increased Crystal violet and Safranin O concentration. Cements with increased PLR have a more compact structure, meaning less free space being present between solid structures due to the increased amount of water in the cement mix. Cements with a high

DISCUSSION

PLR contained less water which was also partially consumed during the setting reaction, which may have resulted in fewer pores formed from air and water trapped in the set structure. Thus, cements with a higher PLR (i.e. PLR 4.0 g/ml) would not allow dye to permeate into the cement structure since the route of dye permeation would be through pores and flaws in the structure of the cement. With this in mind, cements with a lower PLR (i.e. PLR 2.5 g/ml) would have more pores present in the cement structure, providing more routes for dye to permeate into cement, which would explain the increased dye permeation into cements with a low PLR. Porosity decreased with the setting time of the cement due to the water-consuming hydration reactions of PC, producing more well connected cement particles to form a more compact structure with less porosity in the process. Therefore, cements immersed into dye after 10 and 30 days of setting showed considerably lower values for dye permeation compared with samples set for just 1 day before dye immersion.

The visibility of Crystal violet and Safranin O at low concentrations (0.5 %) that had permeated into cements was poor since little contrast was seen between the dye and the grey cement. The high standard deviations of the results obtained for the permeation of dye at low concentrations into cements may reflect the difficulty in judging the point at which the dye stopped permeating into the cement, since more concentrated Crystal violet and Safranin O solutions had results for dye permeation with smaller standard deviations.

India ink did not cause any significant permeation to enable comparisons of the permeation of cements with varying PLR. The size of India ink particles had been compared with those of bacteria, as previously discussed and therefore may stimulate the leakage of bacteria into the cement, however, India ink did not enable the comparison of permeation for cements with varying PLR and therefore cannot be used to characterise changes in the sealing ability of MTA containing various additions of PoP.

9.7.3 Effect of PoP addition on dye permeation into cements

Crystal violet permeation increased slightly with increasing PoP content and decreased drastically with increased setting time of cements prior to dye immersion (see figure 96, section 8.3.1). It can therefore be concluded that the permeation of dye into cement was dependent primarily upon the stage of setting of the cements. Cements containing 30 wt% PoP showed the most dye permeation which would have been expected when compared with the model cements as 30 wt% PoP-containing cements were very porous (see results section 8.3). These pores allow the flow of dye into the cement much more easily which would lead to the dye permeating further into the cement. The dye permeation value recorded in model cements and cements containing 10 wt% PoP were similar when these cements were set for 30 days before immersion in dye for 3 days and those set for 10 days prior to dye immersion. After 30 days of setting, the structure of the cement begins to strengthen further caused by a further reduction in porosity within the cement structure due to the water-consuming reaction forming belite. Therefore, it would be expected that cements set for 30 days before dye immersion would show less dye permeation when compared with those cements stored for 10 days prior to dye immersion. However, the similarity in results could be explained by the fact that the production of belite in cements starts only after 30 days of cements [Taylor *et al*, 1997].

30 wt% PoP-containing cements had significantly more Crystal violet permeation compared with unmodified model cements when set for 10 days. 30 wt% PoP-modified cements had more pores and flaws present in the structure (see section 4.2.2), caused through the rapid setting, which may have provided a route for easier entry of dye into the cement. Gypsum, formed as a result of excess PoP in the cement, could have been washed out of the cement

DISCUSSION

structure on contact with the Crystal violet dye solution that may have left more pores in the cement structure facilitating the entry of dye into the cement.

Unmodified model cements showed the least amount of Safranin O permeation compared with model cements containing 10 or 30 wt% PoP additions which effectively revealed dye permeation throughout the entire sample (see section 8.3.2, figure 97). Safranin O dye may have permeated fully through cements containing PoP because there may have been more pores and flaws present in these cements compared with model cements with no PoP additions, potentially caused by the rapid hardening of cements containing PoP, known as false setting (see discussion section 9.2.2.1). Safranin O and Crystal violet may have travelled into the pores and flaws created during setting of cements containing PoP, causing there to be much dye permeating through 10 and 30 wt% PoP-containing cements when these cements were stored in dye after just 1 day of setting.

Despite the fact there were only small traces of the ink permeated into cements, it was found that India ink was able to permeate into cements similarly regardless of the cement composition. The results may indicate that modified cements may have the potential to provide an adequate seal, preventing the entry of India ink, which would represent bacteria [Schäfer *et al*, 2002] when used in a tooth model. However, since no significant differences between the permeation of ink into modified or unmodified cements were discovered, more sensitive dyes were selected to provide a better comparison of the effect of PoP content on the sealing capabilities of MTA and model cements.

9.7.4 The sealing ability of modified MTA using dye leakage

More dye permeated through MTA modified with 10 wt% PoP, model cements and PoP-modified model cements when compared with unmodified MTA (see section 8.4, figure 98). The indication was that more dye permeated into MTA containing 10 wt% PoP, model cement and PoP-modified model cements due to the hydrostatic pressure, diffusion and a capillary action, forcing the dye into these cements. MTA had a higher density compared with unmodified model cement observed from density calculations (see appendix 2) and SEM photomicrographs of cements (see section 4.5.3, figure 35) may explain the decreased dye permeation compared with model cement because the more compact the cement structure is, the fewer flaws are likely to be incorporated into the cement structure, which could be a pathway for the permeation of dye into cements. However, MTA containing PoP showed the most dye permeation compared with model cements and PoP-modified model cements, which had decreased permeation respectively.

However, MTA and modified MTA with 10 wt% PoP displayed similar sealing capabilities with regard to the dye permeation observed during this study (section 8.4, figure 98). Similarly, model cements with or without 10 wt% PoP revealed comparable sealing abilities in the tooth model and the dye penetration as both did not exceed the sealing capacity of MTA. The similarity of the sealing results confirmed the validity of the model cement for MTA and confirmed that PoP had little effect on the dye leakage into MTA and model cements (to determine sealing ability of MTA and the model cement). PoP had been used in a previous study in conjunction with MTA to seal furcal perforations where PoP also had no apparent effect on the sealing capacity of the root filling compared with MTA without the PoP matrix [Silva Neto *et al*, 2003].

DISCUSSION

It was reported that greater leakage was observed when dye was in contact with the cement immediately when compared with the leakage of dye into cements that had been set for longer periods of time [Torabinejad *et al*, 1995a]. Calcium chloride additions to PC and MTA were reported to enhance the sealing ability of the root filling material. 10% Calcium chloride containing white and grey MTA that were placed in roots and immediately stored in 0.2 % Rhodamine B for 3 days had significantly less leakage of dye compared with white and grey MTA without calcium chloride [Bortoluzzi *et al*, 2006].

Dye leakage studies have been suggested to be unreliable for the assessment of the sealing ability of root filling cements, due to the lack of standardisation of the method and apparent lack of clinical relevance [Veríssimo *et al*, 2006]. However, no standard methods or techniques had been used in the literature when the bacterial leakage method and fluid filtration methods were used for the assessment of the sealing capacity of root filling materials [Veríssimo *et al*, 2006]. No statistical significant correlation was found when the dye leakage method was compared with the fluid filtration method for investigating the sealing ability of root filling materials [Youngson *et al*, 1999]; therefore, the use of dye leakage method was justified by ease of use since no superior method was available. The results obtained from bacterial leakage studies are qualitative rather than quantitative since bacteria that pass through the root filling material could quickly multiply, causing turbidity, indicating that the seal of the root filling material has failed. Therefore, the number of bacteria passing through the filling cannot be quantified [Chailertvanitkul *et al*, 1997, Britto, *et al*, 2003] which provides difficulty in comparing the sealing ability of various root filling materials. It was apparent during this thesis that by controlling the variables of the dye leakage method i.e. by selecting the most reliable dye and appropriate concentration, setting a standard with regard to the time which root filling cements were set before being exposed to the dye and the length of time in the dye, the method could

DISCUSSION

standardised, making comparison of root filling materials easier and more reliable. Since investigating the parameters of the dye leakage method had not been done previously, it would be recommended that the modifications made to the method be used in the future for the investigation of the sealing ability of root filling materials. The comparison of leakage into different root filling cements was assessed with ease to retrieve reliable results once the method had been adapted for reliability.

10 CONCLUSIONS

10.1 *Effect of setting modifiers on mechanical properties of MTA and model cement*

The additions of PoP and CaSO₄ accelerated the setting of PC-based commercial MTA considerably. The incorporation of the setting modifiers, 10 wt% PoP and CaSO₄ to model cements accelerated the initial setting, whilst maintaining a high CS and a low RP which may be due to the improved workability of the cement paste. Gyp did not accelerate the setting of PC and its addition compromised the CS and RP of cements with increasing Gyp additions. The incorporation of 20 wt% CaSO₄ and 30 wt% PoP caused a decreased CS and increased the RP (and thus reducing the potential durability and sealing ability), associated with rapid setting.

PoP and CaSO₄ accelerated the setting of cement possibly due to the high solubility of these calcium sulphates when compared with Gyp. The higher solubility of these calcium sulphates may have caused more calcium and sulphate ions being readily available for the hydration of cement. It may be suggested that with additions of PoP and CaSO₄, large amount of calcium sulphate may become available for the rapid hydration of alite and belite phases in cements, which are associated with rapid hardening, causing an abnormal setting of cement, known as ‘false’ setting. PoP and CaSO₄ setting modifiers therefore have the potential to provide a solution to one of the major drawbacks of PC based dental materials.

The effect of PoP on MTA was similar to that of the model system thus indicating the suitability of the use of the model system. PoP has the potential to provide a solution to the long setting time of MTA whilst maintaining the CS and RP since it did not profoundly affect the mechanical stability of MTA.

10.2 *Ageing of MTA and model cement containing PoP*

The mechanical stability of grey and white model cements containing 5-10 wt% PoP were not significantly affected by long term storage in culture medium but mechanical stability did significantly decrease with time when large additions (30 wt%) of PoP were added to grey and white model cements stored in water. Culture medium storage may provide a more realistic insight into the effect of ageing on model cements containing PoP additions compared with water storage.

10.3 *In vitro effect of PoP on MTA and model cement*

The effect of PoP additions to MTA on cell growth and proliferation had not been fully explained. The addition of 1 day set 30 wt% PoP to set model cements, set MTA and model cement pastes had similar trends for cell density and proliferation as model cements, regardless of cell type had lower cell densities when compared with the cement-free control. PoP additions have potential as setting accelerants for MTA as the *in vitro* response of neonatal and adult BMSCs, primary osteoblasts and periosteal cultures did not differ during the studies conducted when compared with MTA without PoP. A zone of cell lysis was observed surrounding MTA and model cements with or without PoP. It may be concluded that MTA and MTA with PoP additions influenced cells by a similar mechanism to that of calcium hydroxide, which may be due to the release of calcium and hydroxyl ions from model cements with or without PoP. PoP had been reported in the literature to be unlikely to have a detrimental effect on tissue pH by others and PoP was shown to be involved in the regeneration of bony tissues through the release of calcium ions [Orsini *et al*, 2004]. PoP is also likely to encourage faster hydroxyapatite deposition on the surface of MTA, which appeared to be present on model cements containing PoP that were aged

CONCLUSIONS

in culture medium. Therefore, the addition of PoP to MTA is likely to benefit the *in vivo* response to the root filling cement on placement and may strengthen the bond between dentine and the root filling cement, potentially by the regeneration of the involved tissue.

10.4 *Sealing capability of MTA and model cement with PoP*

It has been shown in this study that the dye leakage method for determining the sealing ability of root filling materials includes many parameters that vary significantly, such as the dye and concentration used, the time that the filling material was set for before dye immersion and the length of time stored in dye. Therefore, dye leakage studies may be considered as rather variable considering the parameters of the method that can be varied. However, standardising the method may have provided a better interpretation of the effect of setting modifiers on the sealing ability of MTA and enabled comparison with the literature for the sealing ability of other modified cements. Therefore, the use of 5 % Crystal violet with the methods previously discussed was identified for the use in the dye leakage method for determining the sealing ability of MTA with additives with the aim to have a standard method that used one dye only. MTA and model cements displayed similar sealing capabilities thus sustaining the reason for use of the model cement for MTA. Dye leakage studies using 5 % Crystal violet revealed that the sealing ability of MTA and model cements did not significantly change when PoP was added. Since PoP accelerated the hardening of MTA, an immediate seal may be provided to the root canal, should MTA be used as a root canal filling. An immediate seal will prevent the entry of bacteria and the leakage of tissue fluids that will sustain the longevity of the root filling cement. It can therefore be concluded that PoP addition maintained the sealing ability of model cements and MTA.

CONCLUSIONS

In summary, the studies conducted during this thesis suggested that the addition of 10 wt% PoP improves the overly long setting time of model cements and MTA whilst maintaining the mechanical stability of the material (and longevity when aged with simulated biological fluids), maintains the *in vitro* response and sealing ability of the root filling cement.

11 FURTHER WORK

11.1 *Effects of blood admixing with PoP-modified MTA on ageing*

Admixture of blood with PoP-modified MTA may have an impact on the properties of MTA containing PoP additions due to its likely sight of use; it may come into contact with blood during placement. Culture medium storage gave some insight into the ageing of the cement with time. However, in order to provide an even more realistic look into the *in vivo* situation, the effect of blood admixing (containing plasma and proteins) with PoP-modified MTA is likely to provide valuable impression since MTA would be placed in a root cavity where it may be likely to come into contact with patients' blood, potentially causing a change to the PLR of PoP-modified MTA and MTA alone, thus, it is likely to effect the setting and mechanical properties of MTA, which in turn could change the sealing ability of the material.

11.2 *In vitro effect of MTA with PoP additions on cytotoxicity and cell attachment*

The findings from this thesis agreed with the observations of some studies but clearly did not confirm the findings of others, with the main finding that Portland cement based root fillers definitely caused a toxic response to cells initially. Cell inhibition zones were apparent surrounding MTA which may indicate that *in vivo*, tissues are likely to suffer damage which would cause necrotic tissue and possibly jeopardise the sealing ability of the material. The cells directly surrounding MTA should be analysed further in conjunction with the attachment of cells to MTA since it was shown in the present study and by other researchers [Saidon *et al*, 2003, Miranda *et al*, 2009] that an inhibition zone of cell growth forms due to the high alkalinity of

FURTHER WORK

MTA, a finding that conflicts with studies that suggest cells readily adhere to MTA. The two situations are unlikely to be occurring at the same time, therefore, cell adherence and the inhibition of cell growth surrounding MTA must be investigated to clearly identify the effect of MTA and the effect of additives to MTA. Cell attachment to MTA needs to be investigated further using a more reliable method as this study clearly indicates that very few cells attach to MTA albeit some groups reported higher cell densities often in miraculous combination with reported anti-bacterial properties.

The effect of fresh MTA placed immediately after mixing into cultures should be investigated with regard to cell attachment, growth and proliferation to the setting cement, MTA as this would be more representative of the clinical situation and give an indication of the *in vivo* response of MTA.

The morphology of cells in close contact with fresh and set MTA containing PoP could determine whether MTA containing PoP has any impact on changing cell morphology (by using an SEM) or generating cell differentiation by analysing changes in gene expression. The claim that MTA is bioactive could be clarified by analysing the release of factors associated with dentine and bone regeneration, for example.

11.3 *Sealing capabilities of MTA with PoP using different methods*

Dye leakage studies revealed that the sealing ability of MTA and model cements did not significantly change when PoP was added. The use of dye leakage studies for determining the sealing ability of MTA appears rather arbitrary in the light of this study and has also been questioned by other researchers due to the lack of standardisation of the method. However, since there are many methods employed for the determination of the sealing ability of MTA, the

FURTHER WORK

sealing ability of MTA containing PoP should be investigated further using these other methods which include dye extraction, fluid filtration, bacterial, protein and glucose leakage models. Therefore, results obtained for the sealing ability of MTA containing PoP using these methods would provide a higher level of comparability with the work of others using these methods to study the effect of additives on the sealing capabilities of MTA. The other methods may also yield similar findings as the results obtained for dye leakage studies using a tooth model investigating the sealing capabilities of MTA were unaffected by the additions of PoP.

The sealing ability of MTA containing PoP would need to be monitored with time since the effects of ageing of MTA containing PoP could alter the sealing capabilities of the material with time. Thus dye leakage studies should be conducted for cements aged for up to 1 year of these cements being immersed within a solution to simulate body fluids to see any initial effect of ageing of PoP-modified MTA and its impact on the sealing properties.

Bacteria that have been fluorescently-labelled could be used as a new method to assess the sealing ability of PoP-modified MTA, which would be much more realistic in the way that the bacteria that may enter the root canal filling can be observed with their position of penetration identified within the root filling material. A fluorescent protein-based biosensor was developed that may tag *Escherichia coli* and *Pseudomonas aeruginosa* [Goh *et al*, 2002], bacteria commonly responsible for root canal infections [Peciuliene *et al*, 2008, Chávez de Paz *et al*, 2007]. The number of bacteria could be quantified with the aid of a fluorescent microscope, should the tooth model and root filling cement within the tooth be cut longitudinally into halves for the location of bacteria within the cement structure should bacteria compromise the seal by entry into the cement. If this method was used then it may be enhanced to investigate the antimicrobial effect of PoP-modified MTA using various bacteria responsible for root canal infections.

11.4 *Antimicrobial effect of MTA containing PoP*

The antimicrobial properties of MTA and model cement containing PoP need to be investigated to ensure that the addition does not alter the antimicrobial properties of MTA. The antimicrobial action of MTA is vital for prevention of further root canal treatment and the longevity of the root filling, since bacteria may be likely to enter the root canal during root canal treatment or may still remain following cleaning of the root canal before placement of the root filling cement.

Methods that have been used to determine the antimicrobial and anti-bacterial effect of MTA such as agar diffusion plates (plate used is chosen in accordance with organism being growth on the plate to ensure that growth is not prohibited by the agar plate) such as that used by Estrela *et al*, 2000, seeded with bacteria of choice. The setting MTA paste placed in the centre of the agar plate and incubated. The inhibition of growth surrounding MTA would indicate the antibacterial response of MTA to the bacteria in the study and could be measured for comparison with MTA containing PoP. Should no inhibition of growth be observed on the plate then it is likely that MTA is unable to provide resistance to growth for the bacteria in question. Direct contact test (DCT) could be used also and compared with the use of agar diffusion method. DCT involves measuring the turbidity of bacterial growth after direct contact with MTA using a microplate spectrophotometer since increased turbidity denotes bacterial growth in the broth, therefore little resistance to bacterial growth [Weiss *et al*, 1996].

11.4.1 **Incorporating antibiotics into PoP-modified MTA root filling**

MTA has been reported to be ineffective at inhibiting the growth of some species of bacteria i.e. *Escherichia coli* [Al-Nazham *et al*, 2003] due to the growing resistance of bacteria to

FURTHER WORK

alkaline environments [Peciuliene *et al*, 2008], therefore if antimicrobial research confirms that this is the case and that PoP-modified MTA is also ineffective at inhibiting the growth of these bacteria, then it would be an idea to enhance the initial antimicrobial effect of the root filling material with antibiotics, incorporated into the cement structure since initial antibacterial action may be essential for the longevity of the root filling material. PoP reduced the porosity of MTA slightly when small additions were incorporated (10 wt%). The incorporation of antibiotics such as penicillin which destroys the cell walls of *Escherichia coli*, could compromise the sealing ability of MTA, however, as more pores could be introduced into the cement. CS may decrease and RP may increase with the incorporation of antibiotics, which are unlikely to interlock with cement particles to provide a well interconnected cement structure because antibiotics would not be involved in the setting reaction of the cement. The addition of antibiotics is also likely to affect the setting time of PoP-modified MTA. Therefore, the impact of antibiotics additions to MTA modified with PoP may be likely to prevent the growth of bacteria in the root canal, which is vital for the success of the root filling and the prevention of further root canal treatment, but could compromise the sealing ability of the material through the antibiotics increasing the porosity of the set cement structure.

11.5 *The addition of other setting modifiers to MTA*

The setting of PoP-modified MTA is based upon a false set of the cement which causes the stiffening of the cement paste. Setting modifiers which may chemically accelerate the setting of MTA would be of interest to be used in conjunction with PoP, which may be able to set on placement in the root canal or have a lower initial setting time than PoP-modified MTA but

FURTHER WORK

possess a suitable working time to allow for adequate time to homogenously mix the cement paste and place the cement correctly in the root canal.

Proposed setting modifiers to MTA containing PoP could be such as calcium silicates [Chen *et al*, 2009], soluble silicates, aluminates, aluminium sulphates, formates, carbonates, soluble chlorides [Rottstegge *et al*, 2006], calcium aluminate or calcium sulphate aluminate and calcium fluoride. The effect of adding additional additives to PoP-modified MTA would mean that that the majority of the methods discussed in the thesis would need to be repeated for the newly modified MTA since the addition of any of these setting modifiers with PoP-modified MTA has not been researched previously and therefore little is known about the impact on the setting, mechanical, biological and sealing properties of the material.

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APPENDIX I

1 Selection of bismuth oxide component to model system

Bismuth oxide added to cement	Compressive strength (MPa)	SD	Relative porosity (%)	SD
Bi ₂ O ₃ Sigma	42.1	13.9	20	1
Bi ₂ O ₃ Acros	39.4	15.0	20	1

2 Addition of PoP to MTA and model system

Cement	Amount of additive	Additive	Compressive strength (MPa)	SD	Relative Porosity (%)	SD	Initial setting time (min)	SD	Density (g/cm ³)			
									Dry	SD	Strut	SD
MTA	0 wt%	Control (no additive)	51.3	8.6	19	1	240	5	2.25	0.02	2.79	0.02
	10 wt%	PoP	47.0	11.4	17	1	40	5	2.24	0.02	2.63	0.02
	20 wt%	PoP	50.4	8.9	19	1	25	5	2.11	0.02	2.60	0.02
Model cement	0 wt%	Control (no additive)	58.1	16.3	21	1	240	5	2.12	0.02	2.67	0.02
	10 wt%	PoP	36.9	3.6	25	1	35	5	2.04	0.02	2.71	0.02
	20 wt%	PoP	34.7	8.6	25	1	20	5	2.07	0.02	2.76	0.02

3 Long term stability and solubility of MTA-like cements containing PoP

3.1 Grey model cements with or without PoP

Storage solution	Length of time	Cement type and additive content (wt%)	Compressive Strength (MPa)	SD	Relative Porosity (%)	SD	Dry density (g/cm ³)	Strut	Wet density (g/cm ³)	SD
Water	10 days	Grey model cement	28.9	8.8	23	1	2.07	2.69	2.26	0.06
		Grey model with 10 wt% PoP	33.8	8.8	23	1	2.05	2.67	2.24	0.08
		Grey model with 30 wt% PoP	24.9	6.0	25	1	2.00	2.67	2.20	0.07
	30 days	Grey model cement	34.0	9.1	18	1	2.13	2.60	2.32	0.08
		Grey model with 10 wt% PoP	33.9	10.2	21	1	2.05	2.59	2.22	0.11
		Grey model with 30 wt% PoP	30.0	7.7	23	1	2.02	2.61	2.23	0.05
	3 months	Grey model cement	40.2	10.3	17	1	2.18	2.62	2.32	0.08
		Grey model cement with 10 wt% PoP	49.7	13.1	17	1	2.12	2.54	2.26	0.06
		Grey model cement with 30 wt% PoP	28.6	5.7	24	1	1.97	2.59	2.19	0.08
	6 months	Grey model cement	38.6	6.5	18	1	2.13	2.58	2.32	0.11
		Grey model cement with 10 wt% PoP	43.8	13.5	20	1	2.02	2.52	2.24	0.12
		Grey model cement with 30 wt% PoP	17.1	6.8	30	1	1.83	2.60	2.11	0.11
	9 months	Grey model cement	41.4	7.5	19	1	2.06	2.55	2.25	0.06
		Grey model cement with 10 wt% PoP	45.5	11.8	22	1	1.95	2.50	2.14	0.07
		Grey model cement with 30 wt% PoP	5.4	1.3	42	1	1.52	2.64	1.91	0.04
	12 months	Grey model cement	30.8	9.7	24	1	1.98	2.50	2.21	0.05
		Grey model cement with 10 wt% PoP	40.5	10.7	26	1	1.86	2.51	2.11	0.05
		Grey model cement with 30 wt% PoP	0.9	0.3	56	1	1.16	2.62	1.66	0.07

APPENDICES

Storage solution	Length of time	Cement type and additive content (wt%)	Compressive Strength (MPa)	SD	Relative Porosity (%)	SD	Dry	Strut	Wet	
							density (g/cm ³)		SD	
Culture medium	10 days	Grey model cement	25.9	8.0	23	1	2.10	2.73	2.30	0.08
		Grey model cement with 10 wt% PoP	34.8	9.6	24	1	2.02	2.65	2.24	0.08
		Grey model cement with 30 wt% PoP	27.4	4.3	28	1	1.94	2.71	2.17	0.12
	30 days	Grey model cement	28.6	5.3	20	1	2.07	2.60	2.24	0.05
		Grey model cement with 10 wt% PoP	29.7	9.5	23	1	2.01	2.60	2.19	0.04
		Grey model cement with 30 wt% PoP	25.9	5.5	24	1	1.99	2.62	2.20	0.05
	3 months	Grey model cement	29.0	7.7	20	1	2.11	2.64	2.28	0.07
		Grey model cement with 10 wt% PoP	37.5	18.0	14	1	2.08	2.43	2.30	0.07
		Grey model cement with 30 wt% PoP	28.8	8.4	24	1	1.98	2.61	2.20	0.13
	6 months	Grey model cement	28.1	5.9	17	1	2.23	2.67	2.39	0.08
		Grey model cement with 10 wt% PoP	49.3	14.3	19	1	2.11	2.60	2.29	0.09
		Grey model cement with 30 wt% PoP	24.6	5.6	23	1	2.00	2.60	2.20	0.08
	9 months	Grey model cement	38.9	6.2	15	1	2.25	2.64	2.40	0.14
		Grey model cement with 10 wt% PoP	40.1	4.7	18	1	2.12	2.57	2.29	0.08
		Grey model cement with 30 wt% PoP	21.3	6.1	25	1	1.93	2.57	2.14	0.09

APPENDICES

3.2 *White model cements with or without PoP*

Storage solution	Length of time	Cement type and additive content (wt%)	Compressive Strength (MPa)	SD	Relative Porosity (%)	SD	Dry density (g/cm ³)	Strut density (g/cm ³)	Wet density (g/cm ³)	SD
Water	10 days	White model cement	36.9	9.9	17	1	2.14	2.59	2.35	0.08
		White model cement with 10 wt% PoP	44.0	11.1	24	1	2.02	2.65	2.24	0.11
		White model cement with 30 wt% PoP	28.9	7.3	24	1	2.00	2.64	2.23	0.06
	30 days	White model cement	40.9	11.3	20	1	2.10	2.63	2.23	0.10
		White model cement with 10 wt% PoP	37.7	11.8	23	1	1.99	2.60	2.25	0.09
		White model cement with 30 wt% PoP	33.5	3.8	24	1	2.04	2.70	2.24	0.06
	3 months	White model cement	46.0	7.3	17	1	2.14	2.56	2.31	0.05
		White model cement with 10 wt% PoP	48.3	19.9	23	1	2.01	2.63	2.25	0.11
		White model cement with 30 wt% PoP	34.3	13.0	25	1	1.96	2.63	2.18	0.07
	6 months	White model cement	35.6	14.9	22	1	2.05	2.64	2.22	0.09
		White model cement with 10 wt% PoP	60.2	18.7	26	1	1.93	2.62	2.16	0.12
		White model cement with 30 wt% PoP	27.7	4.1	28	1	1.91	2.65	2.12	0.05
	9 months	White model cement	37.2	17.4	17	1	2.12	2.56	2.24	0.06
		White model cement with 10 wt% PoP	49.8	14.9	25	1	1.95	2.60	2.18	0.07
		White model cement with 30 wt% PoP	19.5	4.8	29	1	1.86	2.63	2.11	0.07

APPENDICES

Storage solution	Length of time	Cement type and additive content (wt%)	Compressive Strength (MPa)	SD	Relative Porosity (%)	SD	Dry density (g/cm ³)	Strut	Wet	SD
Culture medium	10 days	White model cement	32.8	13.2	24	1	2.01	2.65	2.21	0.10
		White model cement with 10 wt% PoP	35.2	12.5	27	1	1.95	2.66	2.25	0.10
		White model cement with 30 wt% PoP	27.6	3.4	25	1	1.98	2.66	2.23	0.08
	30 days	White model cement	41.4	13.0	24	1	1.94	2.57	2.25	0.09
		White model cement with 10 wt% PoP	50.1	12.4	23	1	2.01	2.61	2.27	0.04
		White model cement with 30 wt% PoP	35.1	7.5	21	1	2.12	2.69	2.29	0.07
	3 months	White model cement	33.1	12.3	16	1	2.22	2.66	2.28	0.13
		White model cement with 10 wt% PoP	42.1	14.6	23	1	2.05	2.65	2.30	0.07
		White model cement with 30 wt% PoP	33.9	9.9	21	1	2.11	2.67	2.29	0.08
	6 months	White model cement	42.0	15.3	15	1	2.23	2.63	2.39	0.10
		White model cement with 10 wt% PoP	45.7	12.1	22	1	2.04	2.61	2.23	0.08
		White model with 30 wt% PoP	33.8	5.1	23	1	2.05	2.68	2.27	0.07
	9 months	White model cement	47.1	22.5	17	1	2.19	2.62	2.34	0.09
		White model cement with 10 wt% PoP	53.6	12.7	19	1	2.11	2.61	2.30	0.11
		White model cement with 30 wt% PoP	26.1	7.1	22	1	2.07	2.65	2.27	0.05

4 *In vitro* Effect of model cements containing PoP on adult BMSC cultures

4.1 *Cultures containing 24 h-set cement paste*

Adult BMSCs cultures	Cell viability(%) of BMSCs attached to culture wells or cements		Cell viability(%) of BMSCs in culture medium	
		SD		SD
Control (no cements)	100	1	25	1
Cultures containing the model cement	25	1	25	1
Cultures containing the model cement with 30 wt% PoP	50	1	50	1

4.2 *Cultures containing modified MTA and model cements*

Cultures	Time (days)	Cell viability (%)					
		Total for cells attached to culture wells and cements	SD	Cells on cements only	SD	Cells on culture wells only	SD
Adult BMSCs cultures only (control)	0	100	0				
	2	95	10				
	4	100	0				
	6	100	0				
	8	100	0				
	10	100	1				
	14	100	0				
Adult BMSCs cultures containing MTA	0	100	0				
	2	82	1	100	0	79	2
	6	92	7	83	29	93	8
	10	96	1	100	0	96	5
	14	97	1	0	0	97	5
Adult BMSCs cultures containing MTA with 10 wt% PoP	0	100	0				
	2	74	1	83	29	72	25
	6	88	1	100	0	87	14
	10	99	1	100	0	99	2
	14	97	1	0	0	97	3
Adult BMSCs cultures containing the model cement	0	100	0				
	2	72	1	0	0	72	25
	6	93	35	75	35	94	10
	10	100	1	0	0	100	0
	14	98	1	0	0	98	3
Adult BMSCs cultures containing the model cement with 10 wt% PoP	0	100	0				
	2	93	1	100	0	92	14
	6	90	1	100	0	90	9
	10	100	1	100	0	100	0
	14	99	1	0	0	99	2

5 Sealing Ability of MTA and model cements containing PoP

5.1 *Effect of varying the PLR of model cement on Dye permeation*

PLR	Time stored before dye immersion (mm)	Dye permeation (mm)									
		Crystal Violet				Safranin O				India ink	
		0.5 %	SD	2 %	SD	0.5 %	SD	2 %	SD	100%	SD
2.5 g/ml	1	0.37	0.18	0.84	0.23	2.95	0.11	2.90	0.12	0.05	0.05
	10	0.07	0.04	0.23	0.09	0.07	0.07	2.97	0.12	0.01	0.02
	30	0.09	0.05	0.30	0.10	0.25	0.16	0.12	0.05	0.02	0.03
3.3 g/ml	1	0.13	0.09	0.35	0.17	0.72	0.25	2.28	0.72	0.02	0.03
	10	0.09	0.08	0.15	0.09	0.10	0.09	0.17	0.15	0.01	0.02
	30	0.00	0.00	0.09	0.06	0.09	0.08	0.06	0.06	0.01	0.02
4.0 g/ml	1	0.04	0.04	0.16	0.11	0.49	0.19	0.47	0.28	0.02	0.02
	10	0.04	0.04	0.05	0.04	0.04	0.04	0.06	0.06	0.02	0.03
	30	0.12	0.07	0.09	0.04	0.05	0.05	0.06	0.06	0.01	0.03

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